Technische Universität Braunschweig

Environmental Chemical Analysis Related to Drug Industries:

Method Development for the Analysis of Benzimidazole Anthelmintics and Corresponding Metabolites in Liquid Pig Manure and Manured Soil

Von der Fakultät für Lebenswissenschaften

der Technischen Universität Carolo-Wilhelmina

zu Braunschweig

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

genehmigte

D i s s e r t a t i o n

von Mohammed Mohammed Ahmed EL-Dosoky
aus EL Dakahlia / Ägypten

1. Referent: apl. Prof. Dr. Robert Kreuzig
2. Referent: Prof. Dr. mult. Dr. h.c. Müfit Bahadir
eingereicht am: 21.03.2012
mündliche Prüfung (Disputation) am: 20.04.2012

Druckjahr 2012

Vorveröffentlichungen der Dissertation

Teilergebnisse aus dieser Arbeit wurden mit Genehmigung der Fakultät für Lebenswissenschaften, vertreten durch den Mentor der Arbeit, in folgenden Beiträgen vorab veröffentlicht:

Tagungsbeiträge

EL-Dosoky, M. M., Kreuzig, R.: Liquid Chromatography-Tandem Mass Spectrometry Analysis of Benzimidazole Anthelmintics and Corresponding Metabolites in Liquid Pig Manure. (Oral presentation). Challenges in Modern Analytical Chemistry. The 16th European Conference on Analytical Chemistry, Belgrade, Serbia, 11-15 September, 2011.

ACKNOWLEDGMENT

First of all, whole praise is to **ALLAH**, the lord of all creatures who taught man the whole science and the names of all things.

The present study was carried out in the Institute of Environmental and Sustainable Chemistry, TU Braunschweig, Germany, during the years 2008-2012. First and foremost, I would like to thank my supervisor **Prof. Dr. Robert Kreuzig**. It has been an honor to be one of his Ph.D. students. He has taught me, how good research in environmental analysis can be done and written. I appreciate all his contributions of time, ideas, experience productive and stimulating.

My sincere and deepest gratitude to **Prof. Dr. Müfit Bahadir**, the head of the Institute of Environmental and Sustainable Chemistry, TU Braunschweig, Germany, for giving me the opportunity to perform this work in Germany and for devoting some of his time to read and evaluate this study.

It is my pleasure to express my appreciation and thanks to **Prof. Dr. Peter Winterhalter**, Institute of Food Chemistry, TU Braunschweig, Germany, for accepting to be the chairman of the committee members of examination.

Many thanks to **Prof. Dr. Dr. Ewald Schnug and Dr. Elke Bloem**, Institute of Crop and Soil Science, Julius Kühn-Institut, for giving me the opportunity to use their LC/MS/MS instrument.

Many thanks to **Dr. Leo Van Leemput** from Janssen Animal Health, a Division of Janssen Pharmaceutica NV, Beerse, Belgium, for supplying the chemical substances under study.

I wish to extend my gratitude to the Egyptian Government, Ministry of Higher Education, represented by the cultural affairs and missions sector in the Egyptian Embassy in Berlin for financial support.

My deep thanks to all my colleagues, especially Amjad Al Tarawneh, Arum Widyasari, Susen Hartung, Saptono Hadi, Nguyen Bich Ngoc and all the entire staff members of the Institute of Environmental and Sustainable Chemistry, TU Braunschweig, Germany, for helping, providing a good working environment, working assistance whenever necessary, and for sharing their scientific knowledge. I would like also to express my deepest thank and profound gratitude to my kind wife **Asmaa** and my little kids **Reem** and **Reham**.

Mohammed Mohammed Ahmed EL-Dosoky

Braunschweig, Germany, in March 2012

Dedicated to:
My country (Egypt), soul of my parents,
my kind wife (Asmaa), my kids (Reem and Reham),
my sisters and my brothers

Contents

Abbreviations

ACE	Acetone
ACN	Acetonitrile
Amu	Atomic mass unit
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
APIs	Active pharmaceutical ingredients
bw	Body weight
CAD	Collision affected dissociation
CAS	Chemical Abstracts Service
CE	Collision cell energy
CEM	Channel electron multiplier
CES	Collision energy spread
CID	Collision induced dissociation
CUR	Curtain gas
CXP	Collision cell exit potential
Da	Dalton
DAD	Diode array detector
DC	Direct current
DMSO	Dimethyl sulfoxide
DOM	Dissolved organic matter
DP	Declustering potential
ds	Dry substance
DFT	Dynamic fill time
EC	European commission
ELISA	Enzyme-linked immunosorbent assay
EMEA	European Medicines Agency
EMS	Enhanced full mass scan
EP	Entrance potential
EPA	Environmental protection agency
EPI	Enhanced product ion scan
ESI (+)	Electrospray ionization in positive mode
ESI (-)	Electrospray ionization in negative mode
ET	Ethyl acetate
EU	European Union

FAL	Federal Research Center for Agriculture
FIA	Flow injection analysis
F.R	Flow rate
Gas 1	Nebulizer gas
Gas 2	Vaporizer gas
GC	Gas chromatography
GF	Glass fibere filtre
GMP	Good manufacturing practice
h	Hour
HLB	Hydrophilic lipophilic balance
HPLC	High performance liquid chromatography
IDA	Information dependent acquisition
IDL	Instrumental detection limit
ihe	Interface heater
IS	Ion spray voltage
ISO	International Organization for Standardization
IQL	Instrumental quantitation limit
IUPAC	International Union of Pure and Applied Chemistry
K_{ow}	Partion coefficient between octanol and water
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LOEC	Lowest observed chronic effect concentrations
m/z	Mass to charge ratio
MDL	Method detection limit
ME	Matrix effects
MEC	Measured environmental concentration
MeOH	Methanol
min	Minute
MM	Manure matrix
MOA	Mode of action
MRLs	Maximum residue levels
MRM	Multiple reaction monitoring
MS	Mass spectrometry
ms	Milli second
MS/MS	Tandem mass spectrometry
MSPD	Matrix solid phase dispersion

MQL	Method quantitation limit
NC	Needle current
NOEC	No observed effect concentration
OECD	Organization for Economic Cooperation and Development
Pa	Pascal pressure unit
PE	Polyester
PEC	Predicted environmental concentration
PNEC	Predicted no-effect concentration
Prec	Precursor ion scan
PPPs	Plant protecting products
PTFE	Polytetrafluoroethylene
Q	Quadrupole
QqQ	Triple quadrupole
R	Recovery
RE	Relative errors
RF	Radio frequency
RP	Reversed phase
RSD	Relative standard deviation
rpm	Round per minute
RT	Retention time
SD	Standard deviation
SDB	Styrene-divinylbenzene
SEC	Size exlusion chromatography
SEM	Secondary electron multiplier
SPE	Solid phase extraction
SW	Surface water
STP	Sewage treatment plant
TOC	Total organic carbon
VMPs	Veterinary medicinal products
UHPLC	Ultra pressure high performance liquid chromatography
UVD	Ultra violet detector
WHC	Maximum water holding capacity
WHO	World Health Organisation
WWTPs	Wastewater treatment plants
λ	Wavelength

1. Introduction

Since the beginning of the last century until now, many chemicals have been produced in order to improve human life and make it more comfortable and longer. Within 11 millions of known chemicals, 100.000 compounds are being produced on large scale and approximately 30.000-70.000 from these chemical are in daily use in the European Union (EU). These chemicals are designed and used for different purposes including agricultural and industrial use. Every year, approximately 300 million tons of synthetic compounds are used in industrial and other consumer products. In agriculture, about 140 million tons of fertilizers with several million tons of pesticides are applied every year. Moreover, a wide variety of these chemical compounds offers direct improvements in human and animal health. Based on the application for which these compounds are used, they end up in one of the environmental compartments (Younes, 1999; Schwarzenbach et al., 2006).

1.1 Pharmaceuticals as emerging environmental pollutants

Human health is a priority in the wealthy developed countries and an objective in the developing countries. In the modern society, medication plays an important role to achieve this purpose. Human beings are not only having different benefits of this health care, but also companion and production animals. In addition to pesticides, disinfectants, detergents, and some of the heavy metals as nutritional supplements, pharmaceutical medicines are important for humans and animals to control versatile health problems. Medicines include all chemicals are used for diagnosis, treatment and prevention of human and animal diseases (Daughton and Ternes, 1999).

The active pharmaceutical ingredients (APIs) are organic complex molecules with molecular weights ranging from 200 to 1000 dalton (Da). According to different chemical structures, these compounds have different physicochemical, biological properties and different environmental behaviors. APIs are usually classified into different groups according to their biological action. These groups are divided into different subgroups according to their chemical structures, e.g., benzimidazole compounds within antiparasitic group. Other classifications refer to the mode of action (MOA), e.g., ß-blockers or calcium channel blockers within the group of antihypertensive drugs (Kümmerer, 2008, 2009).

The continuous increase in human population has created a corresponding increase in the applications and consumption rate of these compounds. Consequently, thousands of tons of these chemicals are used every year (Jørgensen, 2000). Concrete data about the total use of pharmaceuticals worldwide are not available (Ternes, 1998; Boxall et al., 2003). According to

the economic conditions, legislation and patient education, applications and consumption rates of pharmaceuticals are variable from one country to others (Goossens et al., 2005; Huttner and Harbarth, 2010).

Among 50.000 pharmaceutical substances were licensed in Germany in 2001 for human and veterinary medicine, 2.700 containing 900 different active substances are approved and frequently consumed with higher consumption rates. The consumption rates for these drugs in Germany range between few kilograms to several hundred tons per year (Löffler and Ternes, 2003; Kümmerer, 2009). Among the veterinary medicines, antimicrobials and anthelmintics compounds are the most widely used pharmaceuticals in conventional animal husbandry (Tolls, 2001; Sarmah et al., 2006).

According to the medical situation and physicochemical properties, pharmaceuticals are formulated in several dosages forms to be suitable for different administration routes and to stay inside the body for a specific time period. During this time, these compounds may be completely or partially metabolized inside the body forming another compounds defined as metabolites (Lienert et al., 2007). In biotransformation processes, substances are metabolized to phase I or/and phase II metabolites. Phase I metabolites are formed through oxidation, reduction or/and hydrolysis. These metabolites have different physicochemical properties such as higher polarity and less lipid solubility than the parent compounds which are favorable to their excretion (Ternes, 1998). In the biotransformation processes the parent drugs usually undergo for deactivation or activation, e.g., in the case of pro-drugs (Ternes, 1915098; Heberer, 2002a). Phase II metabolites are also water soluble and mostly biological inactive. These compounds are formed through conjugation reactions, where the parent compounds may be linked with glucuronic acid, acetic acid, sulfate or amino acid, etc. (Halling-Sorensen et al., 1998). Once these chemicals are excreted from treated humans or animals and reach the environment via different entry routes, the parent compound and its metabolites can undergo further structural modifications giving new compounds. The resulting compounds from biotic (Halling-Sørensen et al., 2000; Trautwein et al., 2008) or abiotic process are named transformation products (Buser et al., 1998b; Poiger et al., 2001; Schulze et al., 2010). These transformation products may be inactive or have similar or higher toxicity as their parent compounds. The latter is well-known for the naproxen phototransformation products of naproxen (Della Greca et al., 2003; Isidori et al., 2005; Straub and Stewat, 2007). Moreover, the regeneration of the parent compounds from phase II metabolites was reported by several researchers (Khan and Ongerth, 2004; Kim et al., 2010b).

1.1.1 Entry routes into ecosystems

The use of versatile pharmaceutical compounds in large quantities in human and veterinary medicine including degradable and/or non-degradable compounds ensures that some of these compounds are reaching the environment. The presence of these drug residues and their corresponding metabolites even at very low concentrations may pose a direct risk to animal and human health or indirect impacts through interfering interactions in different environmental compartments (Schwarzenbach et al., 2006).

The identification of exposure routes of pharmaceuticals is relevant for an accurate estimation of their predicted environmental concentration (PEC). The latter should be determined in system where the highest concentration can be found, especially when there is more than one entry route for certain compounds as consequence of different application patterns (Jørgensen, 2000).

Following the drug administration, mixtures of parent compounds with or without their corresponding metabolites, are excreted in urine and feces and enter the environment via one of the common environmental pathways. Human medicines reach the aquatic environment directly after passing wastewater treatment plants (WWTPs), where they are usually not totally removed (Kolpin et al., 2002; Ikehata et al., 2006). The sludge from these treatment plants are also used as a soil amendment, where the drugs can be released and finally ended on agricultural soils. In case of veterinary medicinal products (VMPs), manure of treated animals is stored in manure tanks for certain times defined on the legislation of each country (Halling-Sorensen et al., 1998; Jørgensen, 2000; Boxall et al., 2003). During manure storage, pharmaceuticals may be partially or completely degraded. Later on, the manure is used for fertilizing agricultural soils. VMPs can be also released directly into soil, when these chemicals are applied to pasture animals and excreted with urine and feces. Moreover, VMP are directly applied to the aquatic environment when these drugs are used as feed additives in fish farming. Due to surface runoff from soil treated with manure or leaching from application sites, VMPs can also reach the aquatic environment (Kreuzig and Höltge, 2005; Kreuzig et al., 2005). Entering of VMPs to the environment as aerosol and dust in addition to direct emissions from treated companion and production animals, disposing of unused or expired pharmaceuticals into the trash, flush them down the drain or with household waste are established as a minor and less relevant entry routes (Hamscher et al., 2003; Abahussain et al., 2006; Kümmerer, 2008, 2009). Although the emissions of pharmaceutical compounds during manufacturing processes are probably low in the United States (USA) and EU, due to very tight regulatory controls and good manufacturing practice (GMP), different situations are present in the other countries. For example, 11 compounds have been found at concentrations > 100 µg/L in the effluent from WWTPs serving about 90

bulk drug manufacturers in India (Larsson et al., 2007). In 1988, oxytetracycline was detected at concentration > 50 mg/L in the effluent of its production facility in China (Li et al., 2008). **Figure 1.1** shows the major and minor pathways of human and veterinary medicines into the environment.

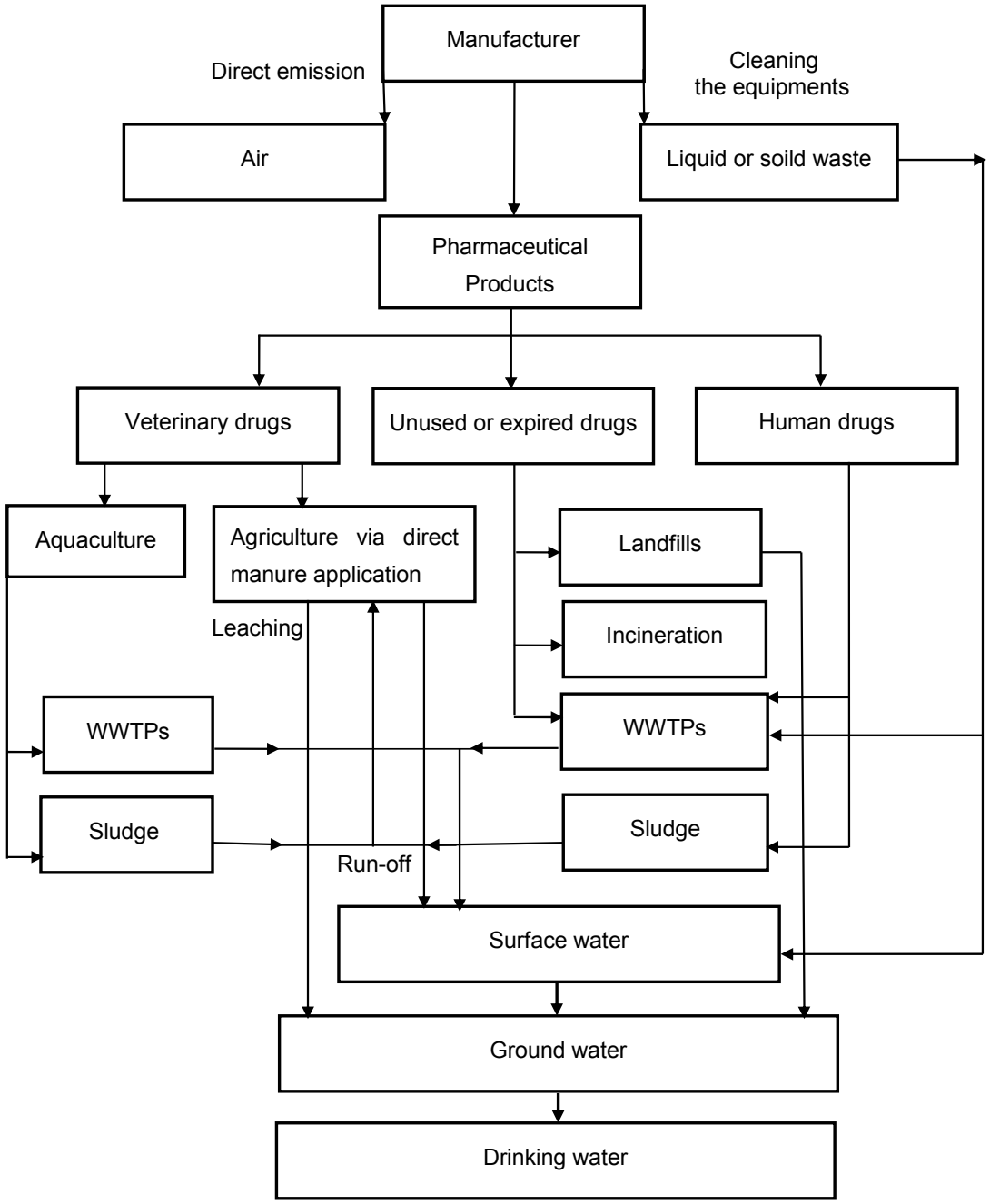


Figure 1.1: Pathways of human and veterinary medicines into the environment.

1.1.2 Occurrence of pharmaceuticals in ecosystems

When salicylic and clofibric acids were detected in wastewater samples at higher concentration levels in 1977, detection of APIs in environmental compartments has become an area of growing concern (Ternes, 1998). During the following years, more awareness focused on pharmaceutical detection in environmental samples and the potential impacts of this new class of environmental pollutants. Since this time, APIs have been detected in different environmental compartments. Pharmaceutical compounds from various classes were detected in wastewater (Hernando et al., 2006; Karthikeyan and Meyer, 2006; Nikolaou et al., 2007; Kasprzyk-Hordern et al., 2008), surface water (Buser et al., 1999; Hilton and Thomas, 2003; Cha et al., 2006), groundwater (Zhu et al., 2001; Batt and Aga, 2005), drinking water (Heberer, 2002b; Ternes et al., 2002), sea water (Samuelsen, 1989; Weigel et al., 2002), sediments (Löffler and Ternes, 2003; Kim and Carlson, 2005), sludge (Nakada et al., 2006; Barron et al., 2008), manure (Haller et al., 2002; Christian et al., 2003; Stoob et al., 2007) and soil samples (Drillia et al., 2005; Thiele-Bruhn and Beck, 2005). The presence of these chemicals in the different environmental compartments may be found with ecological impacts or accompanied with harmful effects on the ecosystem. In this sense, pharmaceutical compounds have been identified as emerging environmental pollutants.

As a result of systematic investigations for the presence of pharmaceuticals in the environment, several pharmaceutical compounds from various classes have been detected in different environmental compartments in different countries, especially when the attention was directed towards the fate and occurrence of these compounds in the environment, e.g., Austria (Ahrer et al., 2001), Brazil (Stumpf et al., 1999), Canada (Miao et al., 2004; Lishman et al., 2006), England (Jones et al., 2002), Germany (Golet et al., 2001; Ternes et al., 2007), Italy (Castiglioni et al., 2006), Spain (Farré et al., 2001), Switzerland (Buser et al., 1998a), The Netherlands (Belfroid et al., 1999) and the USA (Heberer, 2002a; Karthikeyan and Meyer, 2006).

1.1.3 Effects on human, animal and the ecosystems

Pharmaceutical compounds are often present in the environment at low concentrations. Thus, the probability of acute toxicity from these drugs or their metabolites for target and non target organisms is extremely low. This hypothesis is supported by Schwab et al. (2005a). In this study, no risk for human health was observed due to exposure to surface or drinking water containing 26 pharmaceutical compounds at measured environmental concentrations. Han et al. (2006) and Miège et al. (2006) confirmed that the investigated pharmaceuticals

may have ecotoxic effects when environmentally relevant concentrations are exceeded. Two orders of magnitude higher than the highest concentrations of certain compounds in WWTP effluents, was used to study the ecotoxicological effects of these compounds at the lowest observed chronic effect concentrations (LOEC) (Fent et al., 2006). For this reason, the risk to the presence of these compounds in the environment at low concentration has often been assessed as irrelevant effects. However, the possibility of low dose chronic toxicity such as cumulative allergenic, mutagenic, teratogenic or carcinogenic effects which are difficult to assess, may occur after long contact time (Andersson and Skakkebaek, 1999). Numerous studies have been published showing the potential impacts on human, animal and the ecosystem due to continuous exposure to these chemicals in the environment.

Antibiotic resistance and drug tolerance are relevant consequences to a misuse of the drugs, e.g., sub-dosing or occurrence of the drug in environment at low concentration levels in the areas where antibiotics had been intensively used. Humans may be exposed to these resistant strains and then suffered from different health problems (Kim et al., 2005; Ding and He, 2010). Resistance to anthelmintic drugs especially against benzimidazoles has been reported as well (Diiwel, 1977; Hennessy, 1994; Kümmerer, Bauer et al., 2002; 2004; Schwab et al., 2005b).

Some pharmaceuticals, such as estrogens which have the abilities to disturb the endocrine system at low concentrations, are defined as endocrine disrupting compounds (EDCs) (Westerhoff et al., 2005). These can cause several undesirable effects including teratogenic and carcinogenic effects such as breast cancer as well as decrease in human sperm (Ternes et al., 1999). Nash et al. (2004) reported that reproductive failure in fish is resulting from long exposure to environmental concentrations of the pharmaceutical ethinylestradiol. Another example, triclosan is a widely used biocide in hundreds of common commercial products. This compound has been detected in the environment as a contaminant of sewage sludge and water samples (Boyd et al., 2004; Loraine and Pettigrove, 2006). Triclosan has been identified in plasma, urine and human breast milk of people in USA, Sweden and Australia (Dayan, 2007). Triclosan is structurally related to inhibitors of the estrogen sulfotransferase causing negative effects especially for pregnant women and sensitive people, e.g., skin irritation and dermatitis (Andersson and Skakkebaek, 1999; Adolfsson-Erici et al., 2002). Several pharmaceuticals are considered as a risk to the aquatic environment as detailed by Hernando et al. (2006), e.g., acetaminophen, aspirin, ibuprofen, naproxen, diclofenac, ketoprofen, carbamazepine, propanolol, oxytetracycline, ethinylestradiol and estradiol. In his study to assess the potential effects of trimethoprim, ciprofloxacin and mecillinam in the aquatic environment, Halling-Sørensen (2000) found that ciprofloxacin was a highly toxic compound against *Cyanobacteria*, where the ratio of predicted environmental concentration to the predicted no effect concentration was PEC/PNEC > 1. The common case for an

indirect acute ecotoxicological effect which drew the attention of the scientists to direct or indirect impacts of pharmaceuticals in the environment, was the rapid decline of vulture populations on the Pakistan and Indian subcontinent due to renal failure after feeding cattle previously treated with diclofenac (Oaks et al., 2004).

Several experiments have been carried out to assess the ecotoxicity of the veterinary medicines to a group of different organisms such as fish, daphnids, algae, earthworms, microbes and dung invertebrates. In terms of acute aquatic toxicity, the frequently used daphnids and fish appear to be sensitive to the macrocyclic lactones such as ivermectin and eprinomectin. *Cyanobacteria* appear to be sensitive to many antimicrobial drugs such as amoxicillin, benzyl penicillin, sarafloxacin, spiramycin, tetracycline and tiamulin where the effective concentration (EC_{50}) was less than 100 µg/L. For soil organisms, e.g., earthworms appear to be sensitive to antiparasitic and antimicrobial compounds. Those are furthermore reported as highly toxic to soil microbes. Studies on the excreta of production animals treated with macrocyclic lactones show that a highly toxic effect was present to dung invertebrates after prolonged exposure time (Boxall et al., 2003).

Otherwise, the situation may be different in the environment. These micro pollutants are present as mixtures of different compounds with or without their metabolites. Several studies demonstrated that more harmful effect for a mixture of compounds at concentrations at which these chemicals did not have these effects individually (Kümmerer, 2009). In the study performed by Pomati et al. (2006), the effects of a mixture of thirteen human pharmaceuticals on human embryonic cells were investigated at environmental concentrations. It was found that the embryonic cell growth was considerably inhibited. Another study investigated the effect of a mixture of seven pharmaceutical compounds, i.e., acetaminophen, diclofenac, gemfibrozil, ibuprofen, naproxen, salicylic acid and triclosan at 100 ng/L on the amphipod *Hyalella* in freshwater, where slightly changes in sex ratios were reported. This effect increased in areas closer to effluent discharges (Borgmann et al., 2007).

1.1.4 Fate of pharmaceuticals in environmental compartments

Disappearance of APIs in different environmental compartments is mainly attributed to degradation and/or sorption processes. Bacteria and fungi are mainly responsible for the biotic degradation processes where the parent compounds and/or their metabolites may be partially or totally degraded (Jørgensen, 2000; Boxall et al., 2003). Several pharmaceuticals were examined for biodegradability in different environmental compartments. Thus, tylosin was reported as a readily degradable compound, while ivermectin, ceftiofur and metronidazole were described as moderately persistent compounds. Sarafloxacin was even

considered as a highly persistent compound (Boxall et al., 2003). As found in another study, ciprofloxacin and mecillinam were readily degradable chemicals while trimethoprim was persistent compound (Halling-Sørensen et al., 2000).

In the aquatic environment, photodegradation is considered as an additional elimination pathway for photosensitive compounds, e.g., naproxin and diclofenac (Isidori et al., 2005; Schulze et al., 2010). However, the disappearance of these emerging pollutants in the environment does not mean total degradation or depletion so far the pharmaceuticals are merely removed via sorption processes. Sorption is the second major concentration determining process for hydrophobic compounds, e.g., estrogens in activated sludge. Tolls (2001) and Kim et al. (2005) reported that sorption is the main removal mechanism of ciprofloxacin in the activated sludge. In the soil where sludge or manure is usually used as fertilizers for agricultural land, several parameters affect the sorption process such as chemical structure of the drugs, soil type in addition to other parameters such as pH, temperature and ionic strength (Delle Site, 2001). Results published by Kreuzig et al. (2007) demonstrated that sorption to soil is relevant process in benzimidazoles elimination pathways. The decrease of the bioavailable concentration for certain compounds as results of complex formation such as tetracyclines with calcium or/and magnesium in aquaculture was reported as another elimination pathway for these compounds (Lunestad and Goksayr, 1990).

1.2 Anthelmintic drugs

Human and animal are susceptible to parasitic infections. Anthelmintic compounds are a wide range of active ingredients acting against internal parasites via different biological mechanisms. Several anthelmintics drugs are available on the market. These chemicals were classified according to Botsglou and Fletouris (2001) as well as to Lanusse and Pichard (1993) as the following:

Imidazothiazoles

Levamisole is the most common imidazothiazole which has a broad spectrum activity against lung and gut nematodes. Levamisole has no activity against cestodes, trematodes and arthropods. It is frequently used in swine, cattle, sheep, goat, and poultry husbandry.

Organophosphates

These compounds are used as broad-spectrum antiparasitic agents against nematodes and insects. Although, their safety margin is generally less than benzimidazoles, they are used

especially in case of benzimidazole resistance. Haloxon has a wider safety margin relatively than the other members of for this class. It is used in sheep, cattle, and goat husbandry. Coumaphos is used to treat the internal parasites in cattle and swine but dichlorvos is mainly used for swine only to control of gastrointestinal nematodes. Moreover, both of them can be used to control of external parasites.

Tetrahydropyrimidine

Pyrantel and morantel tartrate are used for treatment and control of mature gastrointestinal nematode infections in pig, sheep, cattle and deer.

Salicylanilides

Niclosamide, oxyclozanide, rafoxanide and tribromsalan are the most frequently used salicylanilide anthelmintics to control the liver flukes.

Substituted phenols

The majority of this group has a low safety index and they have been used mainly for treatment of animal liver flukes and tapeworms. These include nitroxynil and niclofolan. Dichlorophen is usually used for treatment of tapeworms beside its bactericidal and fungicidal properties.

Macrocyclic lactones

Moxidectin, abamectin and ivermectin are the commonly used macrocyclic lactones which are mainly active against a variety of nematodes in addition to arthropods, including mites and lice.

Piperazine derivatives

Diethylcarbamazine is one of the piperazine derivatives that can be used in sheep and in cattle husbandry especially for treatment of lungworm infections.

Miscellaneous

Several compounds from different classes are also commonly used as anthelmintic agents such as benzenesulphonamides, e.g., clorsulon is efficiently used for treatment and control of adult and immature liver flukes. Pyrazino-isoquinoline derivatives, e.g., praziquantel, are used to treat many species of cestodes and trematodes.

Benzimidazoles

In 1960s, anthelmintic properties of benzimidazole derivatives were published for the first time. Thiabendazole was one of the first drugs representing this group of anthelmintic agents and licensed for clinical use (Horton, 2000). Because of its antifungal effect, thiabendazole is also used as a fungicidal active substance in various plant protecting products (PPPs) (Danaher et al., 2007). Today, a wide variety of frequently used anthelmintics which have a broad spectrum properties is derived from benzimidazole nucleus, including, thiabendazole (TBZ) analogues for examples cambendazole (CAM) and benzimidazole carbamates, such as albendazole (ABZ), fenbendazole (FEN), oxfendazole (OXF) or fenbendazole-sulfoxide (FEN-SO), flubendazole (FLU), febantel (FEB), mebendazole (MBZ), luxabendazole (LUX), triclabendazole (TCB), parbendazole (PAR), oxibendazole (OXI) and netobimin (NETO) (Danaher et al., 2007). These compounds are mostly less-soluble and exhibit much slower removal rates, increasing their efficacy against several types of cestodes, nematodes and trematodes. Some of benzimidazole carbamates are also used in human medicine such as FLU, ABZ and MBZ. The majority of these compounds are biological active with some exceptions which are manufactured as pro-drugs. These compounds have slightly or no biological activity and developed intentionally to increase water solubility and their administration routes (Gottschall et al., 1990). Febantel converts into active metabolites soon after administration. The main metabolites of this compound are FEN and its metabolites. Benzimidazole compounds expel or kill the parasites via several suggested mechanisms, e.g., blocking glucose absorption of susceptible parasites and also depleting their glycogen reserves. Energy depletion due to decreasing of adenosine triphosphate (ATP) formation leads to starving the parasites to death or paralyzing them by interfering parasite neuromuscular pathways, losing their ability to keep their position in the gut or blood (Frayha et al., 1997; Spasov et al., 1999; Horton, 2000).

1.3 Selection of the target compounds under study

The majority of the above mentioned anthelmintic groups are effective against a single class of helminthes. Otherwise, several benzimidazole compounds are broad-spectrum anthelmintics and can be used for treatment and control of gastrointestinal roundworms (nematodes), lung worms (trematodes) and tapeworms (cestodes). Relevant representatives of those are FEN and FLU which can be used for treatment and control of the infection in cattle, sheep, goats or horses, and dogs, cats or poultry and pigs, respectively. FLU is also used as human medicine in different countries (Stürchler, 1987; Horton, 2000). Moreover,

modified FLU UMF-078, which is used as antifilarial drug in Yemen and 34 Africans countries, is considered as another indirect source for FLU and its metabolite (Ramanathan et al., 1994; Issar et al., 1999; Dec Bronsvoort et al., 2008). FEN and OXF are used as APIs for treatment and control of the infection, these compounds can be found with fenbendazole-sulfone (FEN-OSO) and febantel sulfoxide (FEB-SO) as the main active metabolites of FEB when it is used as deworming agent (Rose, 1999; Botsoglou and Fletouris, 2001). The higher consumption rates of the above mentioned compounds as a result of this wide application range is a primary reason why FEN and FLU together with their corresponding metabolites are expected to be present in the environment. Generic properties, CAS numbers, chemical names, structures, molecular formula and molecular weights (MW) of the target compounds are summarized in **Table 1.1** and **1.2**.

Table 1.1: Fenbendazole and corresponding metabolites.

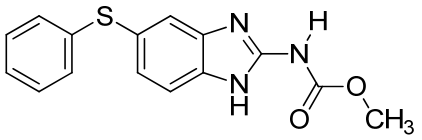
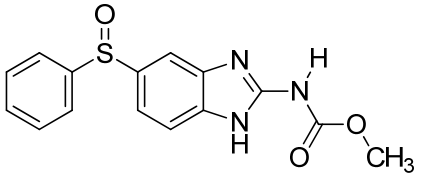
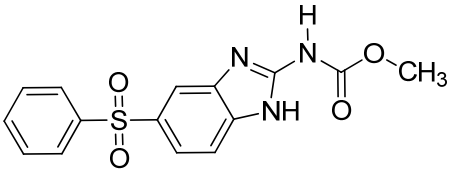
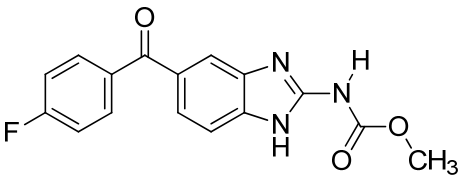
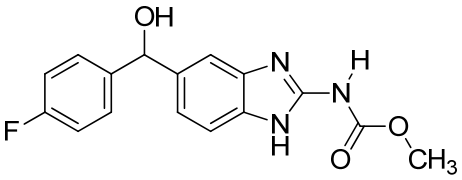
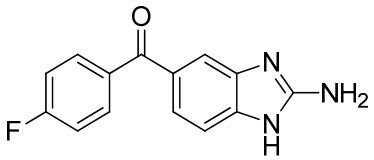
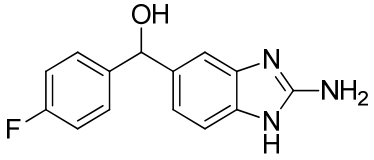
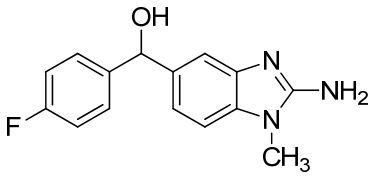
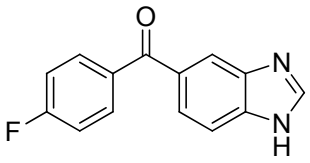
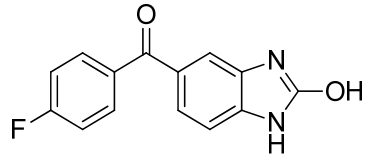
Substance	Chemical Structure
Fenbendazole (FEN) Methyl-[5-(phenylthio)-benzimidazole-2-yl]carbamate ($C_{15}H_{13}N_3SO_2$; MW: 299.3, CAS 43210-67-9)	
Fenbendazole sulfoxide (FEN-SO) Methyl-[5-(phenylthiooxide)-benzimidazole-2-yl]carbamate ($C_{15}H_{13}N_3SO_3$, MW: 315.3, CAS 53716-50-0)	
Fenbendazole sulfone (FEN-OSO) Methyl-[5-(phenylthiodioxide)-benzimidazole-2-yl]carbamate ($C_{15}H_{13}N_3SO_4$, MW: 331.3, CAS 54029-20-8)	

Table 1.2: Flubendazole and corresponding metabolites.

Substance	Chemical Structure
Flubendazole (FLU) Methyl-[5-(4-fluorobenzoyl)-1*H*-benzimidazole-2-yl]carbamate ($C_{16}H_{12}FN_3O_3$; MW: 313.3, CAS 31430-15-6)	
FLU-M1 Methyl-[5-(4-fluorophenyl)hydroxymethyl]-1*H*-benzimidazole-2-yl]carbamate ($C_{16}H_{14}FN_3O_3$; MW: 315.1)	
FLU-M2 Amino-[5-(4-fluorobenzoyl)-1*H*-benzimidazole-2-yl] ($C_{14}H_{10}FN_3O$; MW: 255.3, CAS 82050-13-3)	
FLU-M3 Amino-[5-(4-fluorophenyl)hydroxymethyl]-1*H*-benzimidazole-2-yl] ($C_{14}H_{12}FN_3O$; MW: 257.1)	
FLU-M4 Amino-[5-(4-fluorophenyl)hydroxymethyl]-1-methyl-benzimidazole-2-yl] ($C_{15}H_{14}FN_3O$; MW: 271.1)	
FLU-M5 5-(4-fluorobenzoyl)-1*H*-benzimidazole $C_{14}H_9FN_2O$; MW: 240.1	
FLU-M6 Hydroxy-[5-(4-fluorobenzoyl)-1*H*-benzimidazole-2-yl] $C_{14}H_{10}FN_2O_2$; MW: 256.1	

1.3.1 Synthesis and properties of the target compounds

In the 1950s, the benzimidazole ring system was found as an integral part of the structure of vitamin B_{12} (5,6-dimethyl-I-(α-D-ribofuranosyl) benzimidazole) as it can be seen in **Figure 1.2 A** and **B**. As a result of this finding, extensive studies were conducted to synthesize several thousand benzimidazoles and to study the pharmacological effects of these compounds until the antiparasitic effect was identified for 20 compounds containing benzimidazole ring nucleus. Thiabendazole and its analogues were the first known compounds used for this purpose since 1960s.

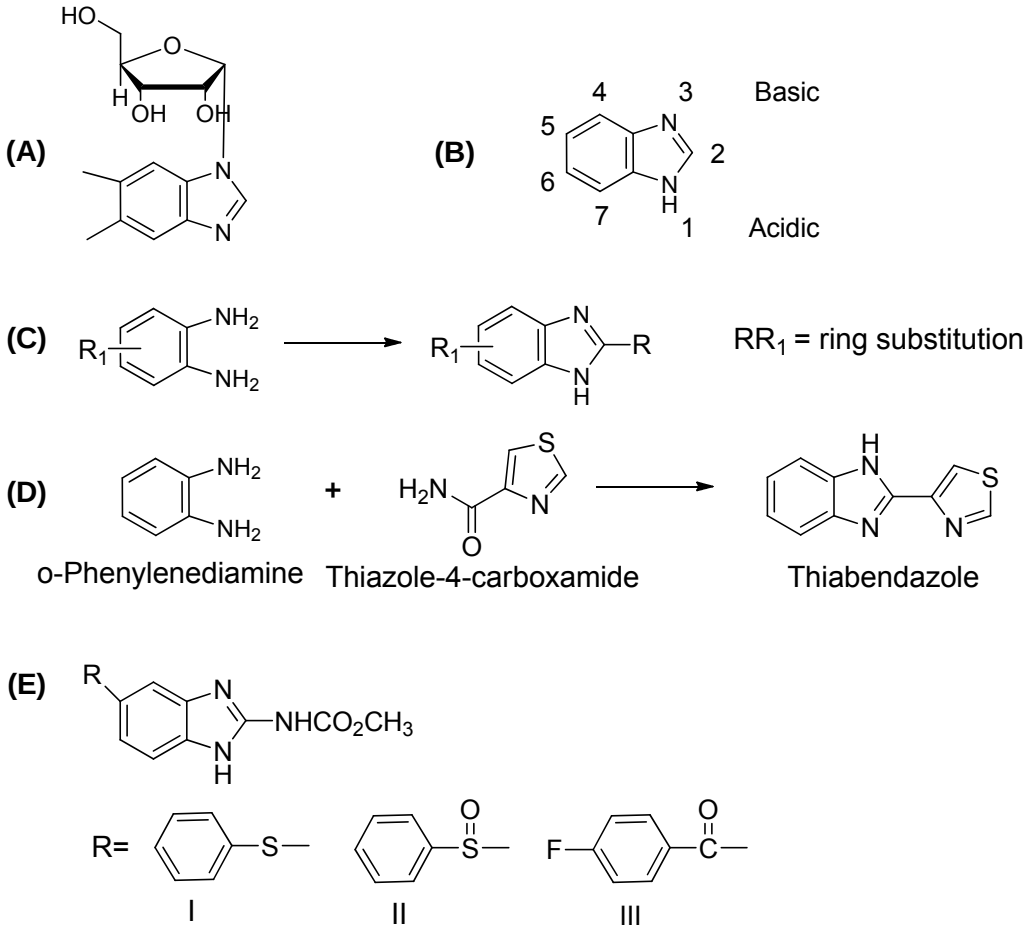


Figure 1.2: Benzimidazole ring system as integrated part of vitamin B12 (A, B) the synthetic pathways to the various benzimidazoles with thiabendazole synthesis as example (C, D) R-ring substitution, e.g., fenbendazole and sulfoxide and sulfone metabolites (E) (Townsend and Wise, 1990).

The synthetic pathways to the various benzimidazoles usually proceed through two steps, first the construction of a benzene ring containing the desired substituent and 1,2-diamine

group followed by the ring closure of the 1,2-diaminobenzene (ortho-phenylenediamine) derivative to construct the imidazole ring. This ring closure is often the final step in the synthesis of the desired benzimidazole (**Figure 1.2 C**).

The second step is the synthesis of the desired benzimidazole modification at the positions 2 and 5, e.g., thiabendazole is synthesized by a condensation of o-phenylenediamine with thiazole-4-carboxamide in the presence of the dehydrating agent such as polyphosphoric acid (**Figure 1.2 D**) (Townsend and Wise, 1990).

A new generation of benzimidazoles with much slower rates of elimination, higher potencies and broader activity spectra than the other antiparasitic drugs are produced nowadays via replacement of the thiazole ring by methylcarbamate at position 2 and different modification at position 5 (**Figure 1.2 E**).

Benzimidazole compounds that are unsubstituted at the imidazole nitrogen atoms possess both acidic and basic characteristics as it is illustrated in **Figure 1.3**. Under suitable conditions, the molecule may be protonated (pK_a < 5) or deprotonated (pK_a > 12). For the better understanding of the behavior of benzimidazole compounds, physical and chemical properties, e.g., octanol/water partition coefficients and pK_a values are essential to explain solubility and ion exchange properties of these compounds in different media at different pH.

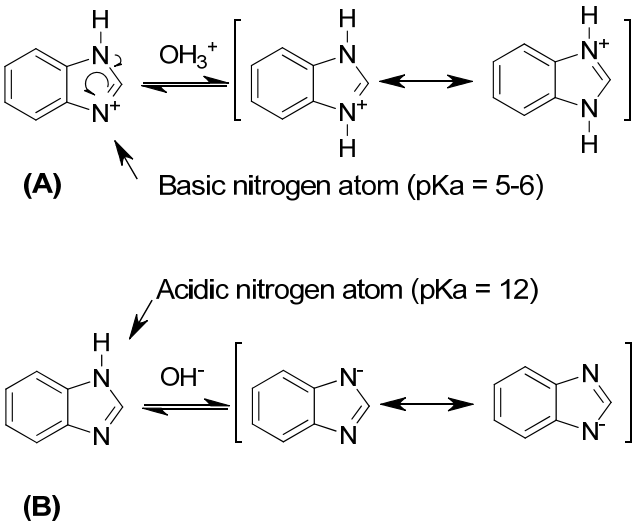


Figure 1.3: Amphoteric charatacter of the benzimidazole molecule under acidic (A) and basic conditions (B) (Danher, 2007; Townsend and Wise, 1990).

The chemical structure of FEN and its pharmacological properties are similar to other benzimidazole compounds. FEN has a broad spectrum of activity against all stages of gastrointestinal nematodes including their larvae, cestodes and lungworms in cattle, goats, horses, cheep and pigs. FEN is known chemically as methyl [5-(phenylthio)-benzimidazole-2-

yl] carbamate. It is insoluble in water (< 40 µg/L) and slightly soluble in common organic solvents such as ethanol, diethyl ether, dichloromethane, n-hexane and toluene. FEN is freely soluble in dimethyl sulfoxide (DMSO). Its log P_{ow} is 3.85 (Kim et al., 2010a).

FLU is a broad spectrum anthelmintic used for treatment and control of the gastrointestinal nematode and lung worms in swine as well as gastrointestinal nematodes in poultry. FLU is known chemically as methyl-[5-(4-fluorobenzoyl) 1-H-benzimidazole-2-yl] carbamate. It is insoluble in water (< 10 mg/L) and diluted inorganic acids as well as slightly soluble in common organic solvents such as ethanol, diethyl ether, dichloromethane, n-hexane and toluene (0.14 g/L). FLU is better soluble in formic acid (340.5 g/L), dimethyl sulfoxide (DMSO) and formic acid (15 g/L) and dimethyl formamide (5.6 g/L). FLU has the ionization constants pK_{a1} = 3.6 (imidazole nitrogen) and pK_{a2} = 9.6 (carbamate nitrogen). Its log P_{ow} is 3.0 (Nobilis et al., 2007).

1.3.2 Pharmacokinetics and metabolic pathways of the target compounds

Several pharmacokinetic studies have been designed to build an overview about absorption, distribution, metabolism, excretion and depletion of benzimidazoles in different animal species. This section is mainly focused on the pharmacokinetic of FEN, FEN-SO and FLU. Pharmacokinetic of FEB and UMF-078 as additional sources for the target compounds are included.

After oral administration, FEN is absorbed rapidly in monogastric and very slowly in ruminant animals. Consequently, the blood levels and excretion half lives are longer in the ruminant than monogastric animals. For examples, absorbed amounts after single oral doses were 25-50 %, < 20 %, 70 %, 25 % and > 33 % for rats, dogs, rabbits, cheep and pigs, respectively. Following absorption, FEN undergoes metabolism by oxidation of its sulfide atom to form FEN-SO, then further oxidation to form FEN-OSO. FEN is metabolized also to other benzimidazole derivatives via hydroxylation of phenyl ring and degradation of carbamate moiety. The resulting metabolites include FEN-SO, FEN-OSO, fenbendazole 2-aminosulfon (FEN-NH_2) and other minor metabolites such as the 4-hydroxy fenbendazole (FEN-OH) in rats, rabbits, dogs, sheep, cattle, goats, chickens and pigs. In the edible tissues of all aforementioned species, FEN, FEN-SO and FEN-OSO are the mainly detected metabolites. In urine, the FEN-OH is the major compound with traces of FEN-SO and FEN-OSO. Finally, more than 90% can be excreted within 3 days mainly with the feces which is considered as the main elimination route (WHO, 1991; Murray et al., 1992).

Several metabolism studies in different animal species have been detailed in the report of WHO (1991) about some benzimidazole compounds. Thus, in a metabolism study in pigs, 3

test animals received 5 mg/kg of 2.5 % oral aqueous solution of fenbendazole. After 3 days, 30-35 % was excreted in the urine while 50-60 % was excreted via feces. 1 % of the parent compound was detected in the urine and 37-52 % was detected in the feces. This finding demonstrated that the absorbed portion of FEN was extremely metabolized in pigs. After 7 day, only 0.28 mg/kg was found in the liver of the pigs treated with single dose of FEN at 5 mg/kg body weight (bw). Another study showed that liver contained 1.3, 1.9 and 0.1 mg/kg of FEN, OXF or FEN-SO, and FEN-OSO, respectively, after 7 day in cattle treated with 7.5 mg FEN/kg bw. In fish treated with FEN, the parent compound and its metabolite FEN-SO can accumulate in the skin. However, both compounds were mostly depleted within 96 and 24 h, respectively.

Unlike FEN, OXF may be administered orally. It is rapidly absorbed in all animals or intraruminally transformed due to its relatively higher solubility than FEN or FLU. OXF is commercially available and widely used in sheep, horses and cattle. As FEN-SO, it is one of the main metabolites of FEN and FEB. Pharmacokinetic data obtained from several studies on different animal species demonstrated that this compound highly absorbed after oral administration, e.g., 100 % in the rats, 77 % in cattle, 85 % in sheep. After administration of the drug to cattle and sheep for treatment and control of roundworms and tapeworms at a dosage of 4.5 mg/kg bw, this compound is rapidly absorbed and then metabolized. FEN, FEN-SO, FEN-OSO and other minor metabolites were detected in the plasma of the treated animals. This finding confirms that FEN-SO again can convert to FEN. The parent compounds and its metabolites mostly are excreted with the feces within 2 days. **Figure 1.4** shows the metabolic pathways of FEN and FEN-SO in different animal species and FEB as another source for FEN and FEN-SO (WHO, 1991; Botsoglou and Fletouris, 2001).

Due to its low water solubility, FLU is a poorly absorbed compound with low toxic effects on the treated animals. Therefore, the oral administration of FLU to pig and poultry is highly effective against gastrointestinal nematodes and lungworms at dosages of 5 or 30 mg/kg bw received in the feed. 50 % of the absorbed amount is excreted unchanged in the feces. Keton reduction and hydrolysis of the carbamate moiety are the major biotransformation pathways in rats, dogs and pigs. Methylation has also been found as minor metabolic pathway in dogs only resulting in the metabolite FLU-M4. Residues of the 2-amino-1H-benzimidazole-5-yl-4-fluorophenylmethanone metabolite were detected in pig liver at much higher concentration than the parent compound, where the absorbed portion is rapidly metabolized. To study the metabolism in pigs, 1.5 mg/kg bw of ^{14}C-FLU was given to 18 pigs where the weight was in the range 16.7 to 24.5 kg to simulate the real dose which is 30 mg/kg. Within 30 days post-treatment, only 79 % was excreted in this period, 56 % with feces and 23 % with the urine.

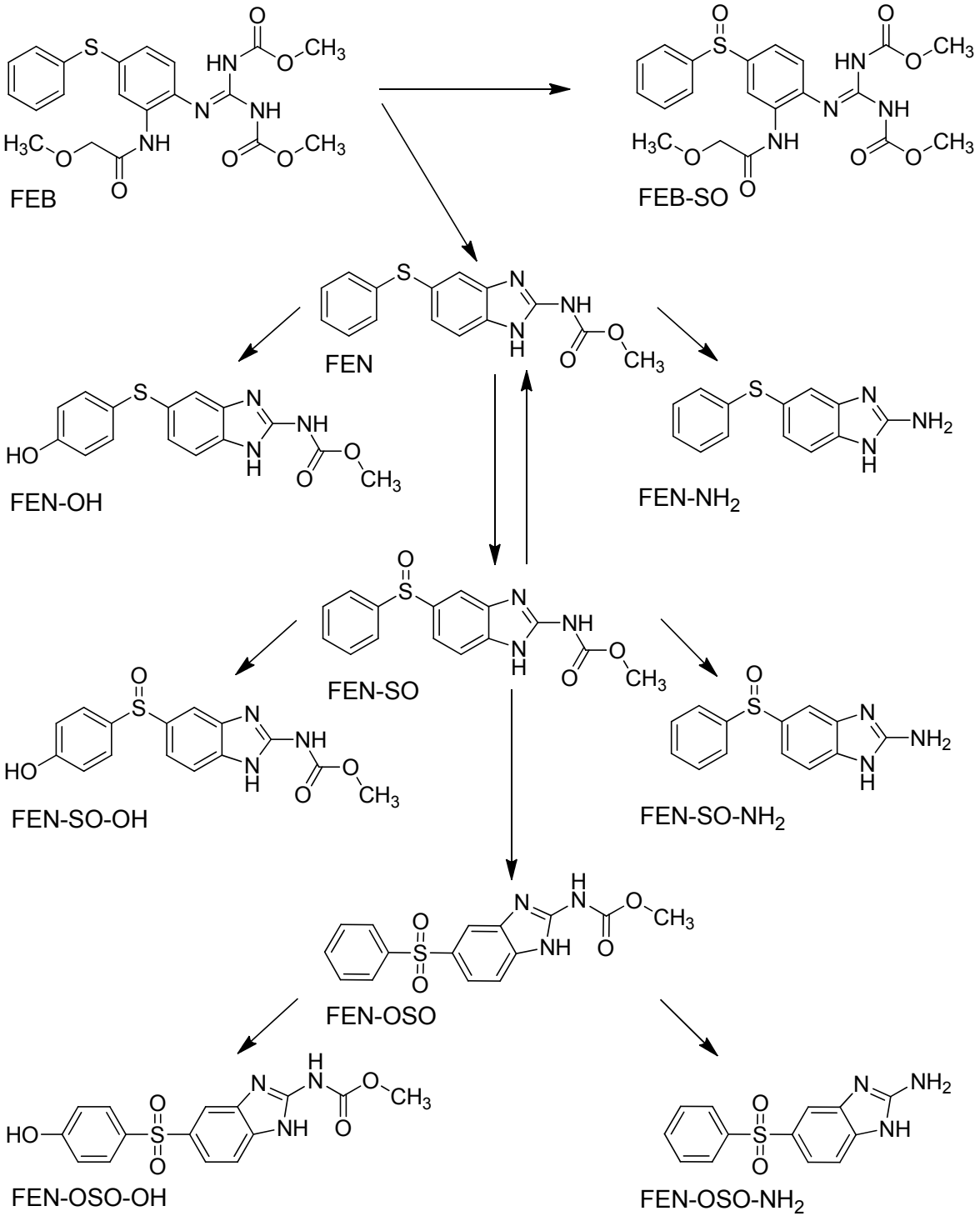


Figure 1.4: Metabolic pathways of febantel and fenbendazole in different animal species (according to Murray et al., 1992; Rose, 1999; EMEA, 2004c).

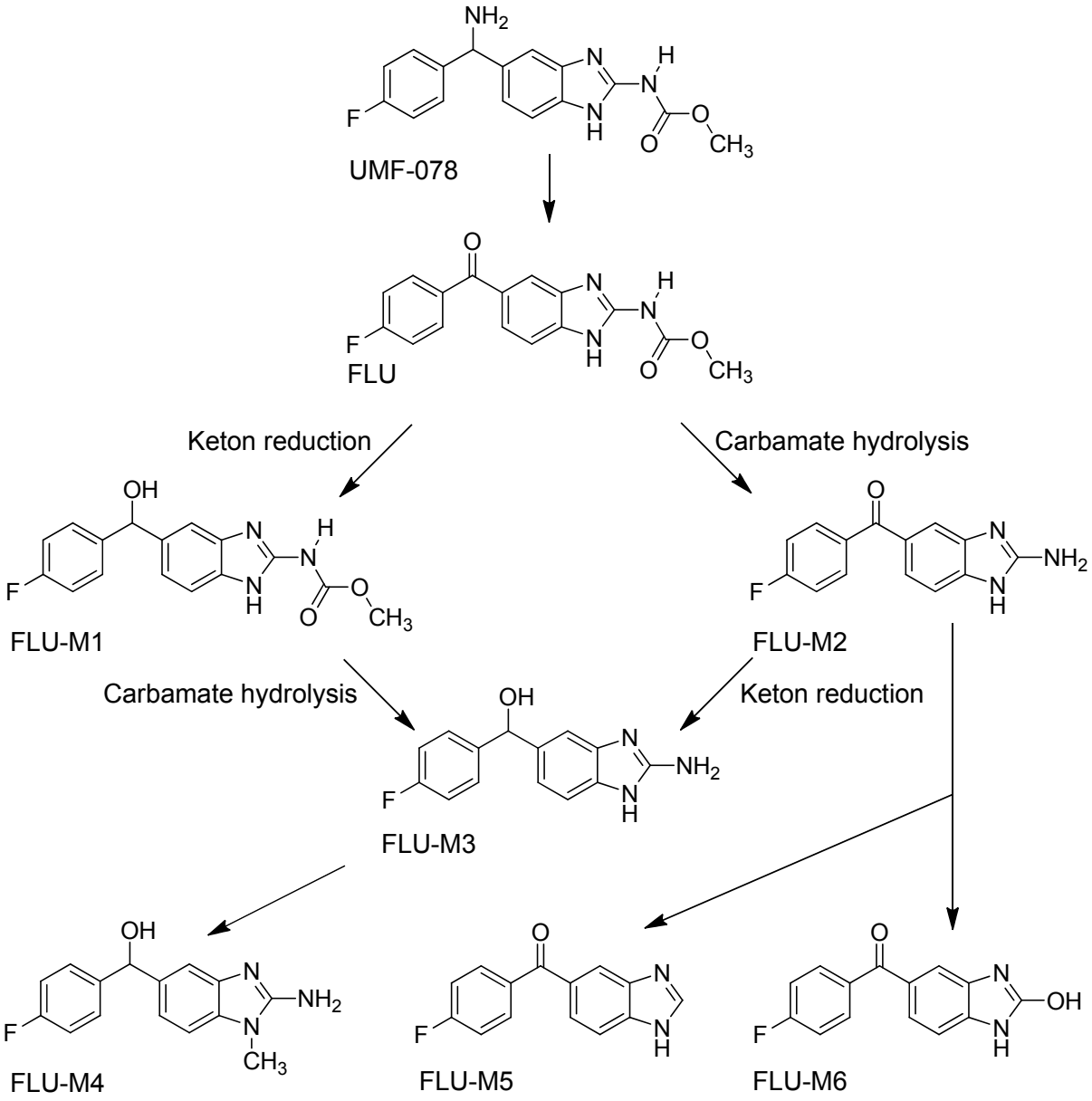


Figure 1.5: Metabolic pathway of flubendazole in different animal species (according to Van Leemput and Heykants 1991).

The third metabolite FLU-M3 resulted from carbamate hydrolysis and keton reduction was the main metabolite detected in the urine. The second metabolite (FLU-M2) which resulted from carbamate hydrolysis was the main one detected in the feces. Otherwise, both of the above mentioned metabolites were detected in pig tissues. FLU-M2 was the main metabolite detected in pig liver tissues. After 30 days, FLU-M2 together with very low amounts of the parent compound less than 2 % were found as well. In another biotransformation study, sows received 30 mg FLU/kg bw in the feed for 10 consecutive days. 7 days after the termination

of the treatment, concentrations of the unchanged FLU in liver, kidney, muscle and fat were 59, 67, 13 and 33 µg/kg bw, respectively. The mean lethal doses reported in toxicological study applied to mice, rats and guinea pigs were > 5000 mg/kg bw. Thus, this anthelmintic drug has a wide safety margin. **Figure 1.5** shows the metabolic pathways of FLU in different animal species and UMF-078 as another source of FLU and its metabolites (WHO, 1993; Issar et al., 1999; Botsoglou and Fletouris, 2001).

Because of these compounds are licensed and applied extensively for food production animals, maximum residue levels (MRLs) of benzimidazoles have been defined for several animal products for human safety by EU. MRLs for FEB and FEN and OXF which are defined as the sum of FEB, FEN and OXF and expressed as FEN-OSO is in the range 10 to 500 µg/kg in milk and different tissues. In case of FLU, MRLs range between 50 µg/kg for muscle and 400 µg/kg for eggs and liver, where the parent compound and the sum of FLU and FLU-M1 are defined in eggs and in other edible tissues, respectively. **Table 1.3** shows the MRLs for FEN, FLU and FEB compounds in selected food products.

Table 1.3: Maximum residue levels (MRLs) for the target compounds (EMEA, 2004a, b, 2006).

Drug	Maximum residue	Animal species	MRL [µg/kg]	Target tissue
FEN FEN-SO	sum of extractable residues oxidizable to FEN-OSO	bovine, ovine bovine ovine porcine equidae	10 50 50 500 50	milk muscle fat liver kidney
FLU	sum of FLU and metabolites	porcine poultry chicken	50 50 400 300 400	muscle skin liver kidney eggs

1.4 Analysis of benzimidazole anthelmintics in different sample matrices

1.4.1 Biological samples

Several methodological approaches were published for the analysis of either single or mixed benzimidazole parent compounds with or without corresponding metabolites. These compounds may be individually analyzed, parent drug plus its major metabolites or parent plus its metabolites mixed with other benzimidazoles. These methods mostly focused on the determination of these selected veterinary medicines in biological samples including urine (Msagati and Nindi, 2006), blood (Ramanathan et al., 1994), plasma (Morovján et al., 1998; Issar et al., 1999; Moreno et al., 2004) and different animal tissues (Brandon et al., 1994; Dowling et al., 2005; Nobilis et al., 2007; Keegan et al., 2011). A number of methods exist for analysis of benzimidazole residues in versatile food types including milk (Brandon et al., 2002; Fletouris et al., 1996; De Ruyck et al., 2002; Hu et al., 2010), eggs (Kan et al., 1998; De Ruyck et al., 2001) as well as animal feed (Dusi et al., 2005; Li et al., 2011). Several compounds were determined in parasitic materials (Mottier et al., 2003; Cvilink et al., 2009) and feces of goat and donkey (Barker et al., 1987; Gokbulut et al., 2006).

Various analytical approaches to sample preparation and detection of benzimidazoles and their corresponding metabolites in different media were published. A number of studies showed that the residues present in urine are mostly present as phase II metabolites. Therefore, de-conjugation of these metabolites via enzymatic hydrolysis was reported as sample pre-treatment step in biological samples (Danaher et al, 2007).

Liquid-liquid extraction (LLE) and solid phase extraction (SPE) procedures were used as efficient methods for extraction and clean-up for benzimidazole analysis. Based on partitioning between two immiscible solvents, several benzimidazole compounds can efficiently extracted. Thus, Fletouris et al. (1996) extracted FEN, FEN-SO, FEN-OSO and FEN-OH from milk samples with dichloromethane after protein precipitation with acetonitrile. This procedure was based on partitioning between aqueous and organic phases at alkaline pH. The organic phases were washed with alkaline buffer and evaporated to dryness and finally the residues were partitioned between water and ethyl acetate.

Blanchflower et al. (1994) developed a method for the determination of FEN and FEN-SO in liver and muscle samples. The samples were homogenized with water, sonicated with methanol and centrifuged. The supernatants were washed with light petroleum. Then dihydrogenphosphate was added and finally re-extracted with diethyl ether/ethyl acetate, where the adjustment of pH was not relevant in the extraction step but later in the clean-up procedure.

Alternative solvents with higher polarity such as acetonitrile and methanol were successfully used without pH adjustment. For example, Sørensen and Hansen (1998) developed a method for determination of MBZ, FEN, FEN-SO and FEN-OSO in muscle and skin tissues. The compounds were extracted with acetonitrile and the extracts were washed with n-hexane before loading on the C_{18} and CN cartridges for additional clean-up. Because of benzimidazole compounds are weakly basic compounds these compounds may be protonated under acidic conditions. Rose (1999) exploited this phenomenon to develop a method for the determination of 9 compounds closely related to OXF in cattle liver samples. The samples were extracted with acetonitrile followed by strong cation exchange SPE for sample clean-up using Bond-ELUT SCX cartridges at acidic pH. The investigated compounds were eluted with acetonitrile containing 5 % ammonia.

A SPE method using styrene-divinylbenzene (SDB1) cartridges has been successfully applied to clean up the raw extracts of pig muscle and liver samples spiked with 15 benzimidazole compounds as described by (Balizs, 1999). These samples were extracted using ethyl acetate at alkaline pH, rotary evaporated and then loaded onto SDB cartridges in methanol/0.1 M ammonium acetate (50:50, v/v). The analytes were eluted with methanol/ethyl acetate (1:4, v/v) and finally analyzed using LC–MS/MS. Recovery of benzimidazole residues was in the range of 36-117 %. Very low recovery of 8 % was obtained for FEN.

Marti et al. (1990) extracted FLU, FEN, FEN-SO and other benzimidazoles from meat by mixing with acetonitrile using a polytron mixer. For every sample, this procedure was repeated one time. The supernatants were removed via centrifugation. The acetonitrile extracts were defatted by stirring with n-hexane and dichloromethane in two consecutive steps. The acetonitrile phase was removed, dried over sodium sulphate and evaporated with a vacuum evaporator. Two more clean-up steps were conducted using Sep-PakC_{l8} cartridges, where the compounds were eluted using acetonitrile. The eluates were passed through Sep-Pak Florisil cartridges and finally eluted using a mixture of chloroform/methanol/triethylamine (90:10:1, v/v/v).

Matrix solid phase dispersion (MSPD) has been applied by a number of researchers for the analysis of selected benzimidazoles in different matrices. A method for extraction of FEN, OXF, FEN-OSO, FEN-OH and other benzimidazole compounds from milk samples was described by Long et al. (1989). The milk samples were mixed with octadecylsilyl (C_{18}, 18 % load, end-capped) derivatized silica packing material. The MSPD column which made from the C_{18}/milk matrix was first washed with n-hexane. The benzimidazoles were eluted with dichloromethane/ethyl acetate. This method was extended to extract the same group of compounds from muscle tissue. The benzimidazoles were eluted with acetonitrile. The

acetonitrile extract was then passed through an activated alumina column for further purification step (Long et al., 1990).

Summary of selected analytical approaches used to determine the benzimidazoles and their related metabolites in different samples is listed in **Table 1.4**. The extraction and clean-up procedures as well as the method detection (MDL) and method quantitation limits (MQL) for different sample matrices are given additionally.

A wide range of analytical techniques were used to determine concentrations of selected benzimidazoles in versatile matrices as detailed in a number of publications, including high performance liquid chromatography (HPLC) with different detectors, such as constant or variable wavelength detectors (UVD) (Nerenberg et al., 1978; Ramanathan et al., 1994; Fletouris et al., 1996; Kan et al., 1998; Rose, 1999; Mottier et al., 2003; Moreno et al., 2004; Dowling et al., 2005; Gokbulut et al., 2006; Nobilis et al., 2007; Kim et al., 2010a) or photodiode array detector (DAD) (De Ruyck et al., 2000), high performance liquid chromatography coupled with mass spectrometry (HPLC/MS) (Barker et al., 1987; De Ruyck et al., 2001; Msagati and Nindi, 2001; De Ruyck et al., 2002; Msagati and Nindi, 2006; Ortelli et al., 2009; Hu et al., 2010; Keegan et al., 2011, gas chromatography/mass spectrometry (Marti et al., 1990), capillary electrophoresis (CE) (Rousseau et al., 2010) as well as a voltammetric method for the quantitative determination of FEN in commercial tablets has been used (de Oliveira and Stradiotto, 2002).

Other techniques offer simple, relatively sensitive and selective methods for detection of benzimidazole residues in biological matrices, such as immunoassay techniques. Nerenberg et al. (1978) developed a method for determination of OXF in rabbit plasma and sheep fat tissue using radioimmunoassay. Using this technique, samples such as plasma, serum and milk can be analyzed directly or diluted prior to analysis. Brandon et al. (1994) determined 11 benzimidazole carbamate residues including ABZ, FBZ, OXI, MBZ, FLU, carbendazim (MBC) and some metabolites in bovine liver samples after aqueous extraction and FBZ residues in diluted milk samples using enzyme-linked immunosorbent assay (ELISA).

1.4.2 Environmental samples

Few data are only available about the analytical determination of benzimidazoles in different environmental compartments especially in liquid manure and manured soil even though these compounds are widely applied in conventional animal husbandry. In the study performed by Van De Steen and Lamber (2008), FLU and 8 other benzimidazoles were determined in surface water and wastewater. Kim et al. (2010a) monitored sorption of FLU, FEN with metabolites and other benzimidazole parent compounds on dissolved organic

matter surrogates and sewage sludge. First transformation tests of FEN and FLU in liquid pig manure and manured soil were performed by Kreuzig et al. (2007). Metabolic dynamics were determined under laboratory conditions by the application of both test substances as ^{14}C-labeled radiotracers. Finally, these results were confirmed under field conditions by means of liquid chromatography with variable wavelength detection (HPLC/UVD). Samples were extracted using a sodium acetate buffer and ethyl acetate and cleaned up by solid phase extraction (SPE) and size exclusion chromatography (SEC). Method quantitation limits were 100 µg/kg liquid manure and 20 µg/kg dry soils. After FLU administration to sows, liquid manures were also analyzed for parent compound and amino and hydroxy metabolites by Weiss et al. (2008). Here, manure samples were only diluted 1:1 in methanol/water (50:50, 1 % acetic acid), centrifuged and LC/MS/MS analyzed at 19-110 µg/L.

Even though, methods for analysis of FLU, FEN and their major metabolites are available in food and biological matrices, only few studies investigated these chemicals in the environment. However, these studies are not sufficient to achieve a complete overview about these compounds simultaneously with their metabolites in the environment and their impacts on different ecosystems and consequently on human and animal health.

Table 1.4: Summaries of selected analytical methods used for the determination of benzimidazole compounds in different matrices.

Analytes	Matrix	Extractant	Clean-up	MDL[a] [µg/kg]	Reference
FLU, FLU-M1, FLU-M2	eggs milk	ethyl acetate at alkaline pH	n-hexane 0.2-µm PTFE filters	0.14-1.14 (LC/MS/MS)	(De Ruyck et al., 2001)
FEN, FEN-SO, FEN-OSO, 12 benzimidazoles	milk	ethyl acetate at alkaline pH	n-hexane SPE/SDB1	< 6 (LC/MS/MS)	(Balizs, 1999b)
FEN, 4 benzimidazoles	muscle milk	supported liquid membrane	supported liquid membrane	10 (LC/DAD)	(Msagati et al., 2001)
FEN, FEN-SO, 7 benzimidazoles	milk	ethyl acetate at alkaline pH	0.2-µm PTFE filters	< 1 (LC/MS/MS)	(De Ruyck et al., 2002)
FEN, 4 benzimidazoles	milk urine	supported liquid membrane	supported liquid membrane	1-10 (LC/MS/MS)	(Msagati and Nindi, 2006)
		SPE (Oasis MCX)	SPE	0.1-1 (LC/MS/MS)	
FEN, FEN-SO, FEN-OSO, 7 benzimidazoles	egg milk muscle	phosphate solution for milk and egg; methanol for muscle	polymer monolith micro-extraction	0.08-2.76 (LC/MS/MS)	(Hu et al., 2010)

Table 1.4: Continued.

Analytes	Matrix	Extractant	Clean-up	MDL[a] [µg/kg]	Reference
FEN, FEN-SO, FEN-OSO, FEN-OH	muscle	PBS-Tween + BSA at pH 7.0	SPE (C18)	3-7 ELISA	(Brandon et al., 2002)
FEN, FEN-SO, FEN-OSO, 3 benzimidazole	muscle	acetonitrile	SPE (C18)	4-26 (LC/UVD)	(Moreno et al., 2005)
FEN, FEN-SO, FEN-OSO	muscle	acetonitrile	SPE (C18, CN)	3.8-4.5 (LC/UVD)	(Sørensen and Hansen, 1998)
FEN, FEN-SO, FEN-OSO, FEN-OH	plasma	acetonitrile chloroform	microfiltration glass fiber	5 (LC/UVD)	(Kellar et al., 2002)
FLU, FLU-M, FLU-M2	egg	ethyl acetate at pH 9.3	0.45-µm PTFE filters	12 (LC/DAD)	(Kan et al., 1998)
FEN, FEN-SO, FLU, FLU-M2, 10 benzimidazoles	liver	phosphate buffer acetonitrile	SPE	5-18 (LC/DAD)	(Caprioli et al., 2010)

Table 1.4: Continued.

Analytes	Matrix	Extractant	Clean-up	MDL[a] [µg/kg]	Reference
FEN, FEN-SO, FEN-OSO, 5 benzimidazoles	animal feeds	acetonitrile	microfiltration	2-63 (LC/MS/MS)	(Li et al., 2011)
FEN, FEN-SO, FEN-OSO, FEN-OH	plasma	acetonitrile chloroform	microfiltration	5 (LC/UVD)	(Gokbulut et al., 2006)

[a] Method detection limit in egg, liver, muscles and animal feeds (µg/kg) and (µg/L) in milk, urine and plasma.

2. Motivation and objectives

Several chemicals have been produced worldwide to maintain the health and yields of food production animals. Besides disinfectant and some of heavy metals which used as food supplements, wide varieties of pharmaceutical compounds are frequently used for treatment and control of several animal diseases. Benzimidazole anthelmintic drugs as a relevant group of the extensively used veterinary medicines are known to be of environmental concern due to their high production volumes and potential adverse effects on non-target organisms in the ecosystem (Danaher et al., 2007; Weiss et al., 2008; Kim et al., 2010a). Three members of this family have been in the focus of numerous research activities due to wide application range as the result of their broad spectrum effect against different parasites, i.e., FEN, OXF and FLU.

In conventional animal husbandry, where the animals are housed and treated with one of selected benzimidazoles for treatment or control of endoparasites, large quantities of solid and liquid manure are produced. The pharmacokinetic studies of selected compounds demonstrated that, following the oral administration, the absorbed amounts are extensively metabolized. The parent compounds and their corresponding metabolites are excreted via feces and urine of treated animals. Excreta are stored for certain time in manure cellars or tanks. Fenbendazole and flubendazole are not readily degraded neither in liquid pig manure nor manured soils based on the reported data about persistence and mobility published by Kreuzig et al. (2007). So far these compounds are not degradable; they will enter soil environments when liquid manures as organic fertilizers are spread to agricultural fields.

Therefore, the occurrence of these compounds in manure and soil in addition to the risk of releasing of these compounds, especially their highly polar metabolites to the aquatic system cannot be excluded. Limited amounts of data are available on concentrations of the parent compounds and their corresponding metabolites in different environmental compartments. This is attributed to the lack of sophisticated analytical method feasible for the simultaneous determination of the parent compounds and their corresponding metabolites in complex sample matrices at µg/kg concentrations. Due to the relevance of the application of benzimidazole anthelmintics in animal husbandry, sophisticated analytical methods are needed. Therefore, the overall objective of the present study focused on the development of an analytical method for the simultaneous determination of fenbendazole and flubendazole as well as their corresponding metabolites in surface water, liquid pig manure, soils and manured soils.

The specific objectives of this work were:

- To develop a chromatographic methods for identification and quantitation of the target compounds.
- To develop sample preparation procedures, e.g., extraction and clean-up methods to allow for LC/MS/MS analysis of the target compounds at µg/kg concentrations.
- To select one of different calibration techniques that allows for compensation matrix effects in order to achieve accurate and precise results.
- To check for the extraction efficiency taking into account aged benzimidazole residues of anaerobic biotransformation tests.
- To evaluate the performance of the purposed methods by the analysis of real manure samples taken from different farms after flubendazole administration to sows and piglets.
- To confirm the positive findings using current standards of analytical quality assurance.

3. Materials and methods

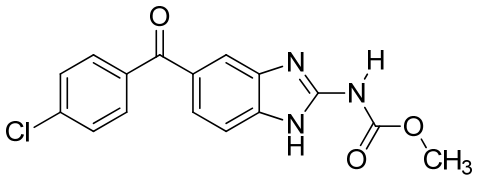
The analysis of benzimidazole anthelmintics in liquid pig manure and manured soil is challenged by versatile factors: Because the parent compounds FEN and FLU, administrated to pigs, and corresponding metabolites formed and excreted by pigs, have to be simultaneously analyzed at low µg/kg concentrations, different structure specific polarities have to be taken into account. Liquid manure and manured soil samples are of high complexity and heterogeneity. Therefore, exhaustive extraction and efficient clean-up procedures are to be developed. Subsequently, liquid chromatography coupled to tandem mass spectrometry follows to exploit its sophisticated selectivity and sensitivity in order to achieve results meeting the current standards of analytical quality assurance. Thus, validation procedures focused on high accuracy and precision, i.e., intra-day and intermediate precision. Finally, positive results were confirmed according to European commission Decision 2002/657/EG (EC, 2002) for identification, quantitation and confirmation of organic pollutant in live animals and animal products.
After the determination of instrumental detection (IDL) and quantitation limits (IQL), therefore, fortification experiments were conducted, first in water, then in reference pig manure and first in soil, then in manured soil in order to identify matrix effects and to determine the method detection (MDL) and method quantitation limits (MQL). Finally, real samples from transformation tests and manure cellars from 7 farms were analyzed to check for the feasibility of the new analytical method.

3.1 Reference standards and standard solutions

FEN, FEN-SO and FLU were purchased from Dr. Ehrenstorfer (Augsburg, Germany). FEN-OSO was synthesized by Kreuzig et al. (2007) via a two-steps oxidation of fenbendazole using peracetic acid as oxidant. FLU metabolites (FLU-M1 to FLU-M6) and the chloro-analogue of FLU (FLU-Cl) (**Table 3.1**) were provided by Janssen Animal Health, a Division of Janssen Pharmaceutica NV, Beerse, Belgium. An individual stock standard solution of each analyte was prepared by dissolving 10 mg of each compound in 10 mL dimethyl sulfoxide. These reference standard solutions were stored at -20 °C and freshly prepared every 3 months. The mixed working standard mixture was prepared in methanol at 50 ng/µL, except of fenbendazole (25 ng/µL) and FLU-M6 (100 ng/µL). The standard solutions were stored at 4 °C and conditioned at ambient temperature before use. Stock standard solution of the FLU-Cl, used as the internal standard was prepared by dissolving 0.1 mg in 10 mL of dimethyl sulfoxide. The working standard solution of 1 ng/µL was prepared by diluting stock standard

solution with methanol and stored at 4 °C. Mixtures of selected compounds at different concentration levels (1 to 50 ng/µL) were used in fortification experiments and for recording the calibration curves. All solvents were of HPLC grade while the other chemicals were of analytical grade. They were purchased from Merck (Darmstadt, Germany). Deionized water was prepared using SERALPUR PRO 90/PRO 90 C Ultrapure water-System with 0.2-µm filter, purchased from SERAL Elrich Alhäuser GmbH (Bansbach-Baumbach, Germany).

Table 3.1: Chemical name, structure, molecular formula and molecular weight (MW) of the chloro-analogue of flubendazole used as internal standard.

Substance	Chemical Structure
Internal standard (IS) Methyl-[5-(4-chlorobenzoyl)-1*H*-benzimidazole-2-yl]carbamate (FLU-Cl) ($C_{16}H_{12}ClN_3O_3$; MW: 329.1)	

3.2 Sampling of different matrices

3.2.1 Surface water samples

Surface water samples were collected using grab sampling method from Oker River at the Botanic Garden, Braunschweig, Germany. These samples were stored in brown-glass bottles pre-rinsed using ethyl acetate and methanol plus deionized water and surface water from the sampling site. Every sample was filtered using 0.6-µm glass fiber filters (GF-6, 0.6 µm; Macherey-Nagel, Düren, Germany) and stored at 4 ºC until analytical processing.

3.2.2 Excreta and real manure samples

Liquid tank manures are well-known to be heterogeneous and complex, already revealed by variable dry substance contents of 0.4-12 % (Burton and Turner, 2003; Merkel, 2005). For experimental purposes, therefore, Kreuzig et al. (2007a; 2010a) developed the reference manure concept. Excreta of pigs individually kept in an experimental animal house under defined feeding conditions were sampled and conditioned. Subsequently, tap water was added to adjust dry substances contents of 5 %. Following this concept, a representative and

reproducible manure matrix, free of interfering veterinary medicines administrated to pigs and biocide, i.e., cleaning and disinfection agents, applied in animal houses, was available for analytical methods development (Kreuzig, 2010a).

For this reason pig excreta, i.e., urine and feces containing 11.1-15.6 % dry substances (ds), were sampled at the Institute of Animal Nutrition, Friedrich-Löffler-Institut, Braunschweig, Germany. The excrement samples were stored in 20-L plastic containers at ambient temperature for 21 days. Within this conditioning period, they were homogenized every 2 days using an electric stirrer and matrix characterized, i.e., dry substances (ds), total organic carbon (TOC) and pH. Bulk excreta were thereafter divided into aliquots and stored in 1 to 2-L plastic containers. These samples were stored at -20 ºC. One of these samples was kept at 4 ºC and analyzed for background contamination. Subsequently, these matrix characterized and background analyzed samples were used in the fortification experiments.

Real manure samples were taken at 7 pig fattening farms in the catchment area of the Chamber of Agriculture of Lower Saxony, Oldenburg, Germany. As broad spectrum antiparasitic agent, FLU was administered as a food additive at 5 mg/kg bw for 5 consecutive days. On 6 June 2010, 10 to 80 days after FLU administration, grab sampling was conducted in manure cellars under the slatted floors. In order to check if FLU and corresponding metabolites can be determined, 500-1000 g real samples were taken with beakers, transferred into polyethylene bottles and cooled down for the transport to the laboratory. The samples were matrix characterized for dry substance, pH and total organic carbon and finally frozen at -20 °C until analysis.

3.2.3 Soil samples

Two different soils were used for this method development. First, samples of humid silty sand soils were collected from the topsoil (0–20 cm) of an arable field at Julius Kühn-Institut, Braunschweig, Germany. Silty clay soils were collected from arable land at Adenstedt, Ambergau, Germany. The field fresh soil samples were homogenized, sieved to < 2 mm and stored at -20 °C until analytical processing.

According to the maximum amount of manure, based on 170 kg N/ha with 5 g N/kg manure, 1500 kg soil/m^3 and 0.05 cm soil depth, that could be added to the soils as fertilizer and accepted by the German Ordinance Concerning Fertilizers (2006), manured soil samples were prepared by adding 10 g of pig excrements to 50 g soil.

3.3 Matrix characterization

3.3.1 Excreta and real manure samples

3.3.1.1 Determination of pH and dry substance

In accordance with DIN EN 12176 S5 (1998), the pH values were measured directly in the excrements by means of pH-meter (Multical 535 GLP with pH-glass electrode SenTix 61; WTW, Weilheim, Germany). Afterwards, reference manure samples were measured to adjust the pH as required during the work. The pH meter was calibrated and tested before every measurement using three different buffer solutions (pH 4.0, 7.0, 9.2). These solutions were freshly prepared if any turbidity could be observed or at maximum 3 months from the first use. The reading was recorded after getting stable for 2 min.
According to ISO 11465 (1993), dry substance percentage (ds) was determined by calculating the difference between the weight of sample before and after drying using Ultra-X infrared heater (Gronert, Lage, Germany). The dry substance content was calculated according to:

$$ds = \frac{m_b}{m_a} \cdot 100$$

Where,
m_a: initial weight (g)
m_b: output weight (g).

3.3.1.2 Total organic carbon

The total organic carbon (TOC) was determined in accordance with ISO 10694 (1995). Amounts of well mixed pig excrement samples (10-20 g) were oven-dried at 105 °C. To remove the inorganic carbon such as bicarbonate, samples treated with 5-20 mL of 4 M of hydrochloric acid (HCl) 2 h or until no more effervescence was observed. During this time, the excess amount of HCl was removed by heating using hot plate at 100 ºC. Samples re-dried in drying oven at 105 °C overnight. After cooling, samples were ground and prepared for TOC analysis (C-Analyser Dohrmann DC-90; Santa Clara, CA, USA). Because of higher carbon content of manure samples, these samples were diluted with aluminum oxide in ratio of 1:19. The mixed samples were combusted at 900 ºC and the combustion gas was ana-

lyzed by means of a non-dispersive infrared detector. The TOC analyzer used for detection of carbon dioxide was operated and controlled by HP-PEAK 96 software Agilent, Waldbronn, Germany.

Samples of low TOC contents were measured without any dilution. TOC amounts were calculated on the basis of an external standardization. The standard curves (20 to 180 µg carbon ranges) were recorded by analyzing mixtures of oxalic acid dihydrate and aluminum oxide ($H_2C_2O_4/Al_2O_3$) at the ratio of 1:9. Finally, the results were calculated as the percentage of the amount (%) of dry substance according to the following formula:

$$TOC\ [\%] = \frac{W_b}{W_a} \cdot 100$$

Where;

W_a: initial weight (µg),

W_b: amount of carbon (µg).

3.3.2 Soil samples

Matrix characterization of soil samples is a relevant process for understanding of the behavior of organic pollutants in soil or manured soils samples. **Table 3.2** presents selected physico-chemical properties of soils under study.

Table 3.2: Selected physico-chemical properties of the soils under study (Kreuzig et al., 2007; Achtenhagen and Kreuzig, 2011).

Soil	Silty sand soil (Braunschweig, Germany)	Silty clay soil (Adenstedt, Germany)
Sand [%]	47.0	5.0
Silt [%]	46.7	56.0
Clay [%]	6.3	39.0
TOC [%]	0.7	1.6
WHC_{max} [%]	34.2	43.0
pH $(CaCl_2)$	5.6	6.9

TOC: Total organic carbon, WHC_{max}: maximum water holding capacity

3.4 Extraction procedures

3.4.1 Surface water samples

The preliminary experiments were conducted to develop a method for simultaneously extraction of the target compounds from surface water by solid phase extraction (SPE). In order to optimize the extraction methods, the performances of two different SPE cartridges with polymeric sorbent materials were checked at different pH values of the samples. SPE experiments were conducted using styrene-divinylbenzene (SDB1, 200 mg, 6 mL; Mallinckrodt Baker, Griesheim, Germany) and Oasis HLB (60 mg, 3 mL; Water, Millford, MA, USA) cartridges. The cartridges were conditioned with 5 mL methanol, followed by 5 mL deionized water in case of neutral samples or by 5 mL acidified water (pH 2.3) prepared by adding 5 mL formic acid in 1 L deionized water in case of acidic samples. For SPE, 200 mL surface water from Oker River were spiked with analytes at 5 and 25 µg/L and 0.1, 0.2 and 1 µg/L for HPLC/UVD and LC/MS/MS experiments, respectively.

The surface water samples (200 mL) were filtered using 0.6-µm glass fiber filters (Macherey-Nagel, Düren, Germany) and then acidified before the extraction with formic acid to pH 2.3 checked by a pH Meter (Multical 535 GLP with pH-glass electrode SenTix 61; WTW, Weilheim, Germany). The pH meter was calibrated and tested before measurement with standard buffer solutions prepared at pH 4.0, 7.0 and 9.2 (Fluka Chemie GmbH, Buchs, UK). The water samples forced through the cartridges at a flow rate of approximately 4 mL/min. Subsequently, the loaded cartridges were rinsed with 5 mL deionized water and then air dried under low pressure for 10-15 min. To test the behavior of selected compounds in a neutral water matrix, 200 mL water samples spiked with the analytes at 1 µg/L were extracted directly without pH adjustment. The elution of the retained analytes was performed with 2 x 5 mL methanol or methanol acidified with 1 % formic acid. The eluates were evaporated to dryness under a gentle nitrogen stream and re-dissolved in 1 mL methanol. Finally, 10 µL were injected to HPLC/UVD (λ = 280 nm) or LC/MS/MS, respectively. For determination of the absolute recovery during the SPE procedure, calculated concentration of the target compounds spiked in the water samples before SPE were compared with those obtained in spiked extracts after SPE according to the next equation (Yang et al., 2004; Kim and Carlson, 2007).

$$\text{Recovery} = \frac{\text{Concentration of the samples spiked before extraction}}{\text{Concentration of the samples spiked after extraction}} \cdot 100 \qquad \text{(Eq. 3.1)}$$

3.4.2 Manure samples

Three different procedures were applied to extract the target compounds from liquid manure samples. For the sub-sampling procedure, pig excrements were manually stirred continuously to avoid sedimentation as far as possible and to get representative samples.

3.4.2.1 Direct solvent extraction

Several extraction conditions for FEN, FLU and their corresponding metabolites from liquid pig manure were tested in the preliminary experiments which began with modification of the procedure described by Kreuzig et al. (2007). Four replicates, each one of 50 g reference manure of 5 % (ds), were transferred into 300-mL Erlenmeyer flasks. The samples were fortified with the target compounds at 100 µg/kg fresh sample and extracted without any pH adjustment (original pH 6.8). Manure samples were extracted with 100 mL ethyl acetate by shaking on horizontal shaker (Type 3020, Gesellschaft für Labortechnik, Burgwedel, Germany) at 220 rpm overnight.

The organic phases were transferred into round bottom flasks using folded filter papers (MN615 ¼. ∅150 mm; Macherey-Nagel, Düren, Germany). The extracted samples were subsequently rinsed with another 100 mL for 60 min and finally with 50 mL ethyl acetate for 30 min. The combined extracts were filtered using the same folded filter papers used before with anhydrous sodium sulfate. To minimize the losses during this procedure, both collecting flasks and filter papers with sodium sulfate were rinsed with 25 mL ethyl acetate. The extracts were rotary evaporated to near dryness. The residues obtained from the extracts were re-dissolved in 10 mL methanol, filtered using 0.2-µm polyester filters (Chromafil Type 0-45-15; Macherey-Nagel, Düren, Germany), cleaned-up and finally LC/MS/MS analyzed. The efficiency of the extraction procedure was evaluated at different spiking levels of the target compounds, i.e., 4, 50, 100 and 500 µg/kg fresh manure.

Two series of experiments were carried out to check the effect of the pH on the extraction efficiencies. At acidic pH, four replicates, each one contains 50 g reference pig manure of 5% (ds), were transferred into 300-mL Erlenmeyer flasks. These samples were spiked with target compounds at 100 µg/kg fresh manure and then acidified to pH 4.4 by adding 0.25 mL formic acid. After adding 100 mL ethyl acetate, the flasks were shaken on a horizontal shaker at 200 rpm overnight. The organic phases were removed via filtration. The previously extracted samples were rinsed twice, cleaned-up and analyzed as described before.

The already discussed extraction procedure was additionally performed at pH 9.5 due to the basic character of the benzimidazoles under study. Thus, manure samples were adjusted

using ammonia/ammonium chloride buffer. The latter was prepared by dissolving 33.5 g ammonium chloride in 150 mL water and adding 42 mL concentrated ammonia. Finally, this solution was diluted with deionized water to the final volume of 250 mL. The buffered samples were extracted, cleaned-up and finally LC/MS/MS analyzed. Spiking levels were 2, 20, 100 and 200 µg/kg fresh manure, respectively.

In order to advance the extraction efficiency at alkaline pH, the addition of sodium chloride (10 g NaCl), and the number and time of extraction cycles were tested as well. To test the effect of shaking time during the first extraction cycle, six samples were spiked with target compounds at 100 µg/kg fresh samples. These samples were extracted for one time by shaking independently with 100 mL ethyl acetate at 220 rpm and ambient temperature for 2, 4, 6, 8 (n=1) and 12 h (n=2). The previously extracted samples were rinsed via shaking with 100 mL of ethyl acetate for 60 min and finally with 50 mL of the solvent for 30 min. The obtained recoveries were compared with the recovery of zero samples equally treated, but, spiked after extraction with analytes at the same concentrations. Further two rinsing steps of the same extracted samples were achieved via shaking at 220 rpm with additional 100 mL ethyl acetate for 60 min and finally with 50 mL for 30 min.

3.4.2.2 Liquid-solid extraction after lyophilization

Different series of experiments were conducted to assess the efficiency of several solvents to extract the target compounds from lyophilized manure samples. For this purpose, 50-g liquid pig manure samples (4 replicates) of 5 % dry substance were transferred to 300-mL Erlenmeyer flasks and then spiked with the target compounds at 100 µg/kg fresh manure. These samples were frozen at -20 ºC for 8 h and then lyophilized at -50 ºC and <1 Pa for 18-24 h. Lyophilized samples were extracted using methanol and rinsed twice as described for direct solvent extraction. The samples were shaken on a horizontal shaker (Type 3020, Gesellschaft für Labortechnik, Burgwedel, Germany) at 220 rpm overnight and rinsed twice by shaking with 100 mL methanol for 60 min and finally with 50 mL for 30 min. The supernatants were decanted and filtered using the folded filter papers with anhydrous sodium sulfate. For minimizing loss of the analytes, both collecting flasks and filter papers with sodium sulfate were rinsed with 25 mL methanol.

The extracts were rotary evaporated nearly to dryness. Afterwards, the residues were redissolved in 10 mL methanol and filtered using 0.2-µm polyester syringe filters and cleaned-up. Then, 7 µL of these solutions were injected into LC/MS/MS. Additionally, different solvents such as ethyl acetate, acetonitrile and acetone were tested to evaluate their extraction efficiencies.

Further rinsing for already extracted samples were carried out in 3 consecutive steps after entire extraction procedure as detailed before to ensure that exhaustive extraction was achieved by the first three extraction cycles. Different spiking levels were used to test the extraction efficiency of the purposed method. The lyophilized samples were spiked with the analytes mixture at 4, 10, 50 and 100 µg/kg fresh manure.

3.4.2.3 Ultrasound-assisted solvent extraction

Direct solvent extraction of liquid pig manure

Four samples, each one of 50 g reference manure, were transferred into screw-top Teflon covered centrifuge tubes and spiked with the target compounds at 100 µg/kg fresh manure. The centrifuge tubes were manually shaken for 1 min. The samples were acidified using 0.25 mL formic acid (pH 4.4) and sonicated (2020/240 W HF power at 35 kHz-Sonarex RK 5125; Bandelin electric, Berlin, Germany) for 10 min. These samples were centrifuged at 4000 rpm for 15 min (Megafuge 1.0; Heraeus Instruments, Hanau, Germany). The aqueous phases were decanted and filtrated through 0.6-µm glass fiber filters. The manure samples were rinsed 3 additional times with 50 mL deionized water acidified with formic acid to finally receive 200-mL aqueous extracts. After homogenization by manual shaking, 50 mL of collected aqueous phases were further diluted to 200 mL using acidified deionized water and extracted using SDB 1 cartridges. The SPE cartridges were conditioned prior to sample extraction with 5 mL methanol and equilibrated with 10 mL acidified deionized water. Aqueous manure extracts (200 mL) were adjusted to pH 2.3 with formic acid and enriched on SDB 1 cartridges. The samples were forced through the SPE cartridges at a flow rate of 3-4 mL/min. Subsequently, the loaded cartridges were rinsed by 5 mL deionized water, dried under low pressure for 10-15 min and finally eluted with 2 x 5 mL methanol.

The eluates were evaporated nearly to dryness, re-dissolved in methanol, and then 7 µl of these solutions were injected into LC/MS/MS. Additionally, the solids were preconditioned by freezing at -20 ºC, lyophilized for 4 h and extracted using methanol as extractant and cleaned by size exclusion chromatography only because these manure samples were already washed 4 times so the final extracts were relatively clean. Finally, 7 µL of these solutions were injected into LC/MS/MS.

Liquid-solid extraction after lyophilization

Eight lyophilized samples, each one contained 2.5 g of dry substance, were used to check the efficiency of this technique to extract the target compounds from lyophilized manure samples. Four samples (S1, S2, S3 and S4) were spiked with the target compounds at 100

µg/kg fresh manure prior to lyophilization. The spiked and unspiked samples (B1, B2, B3 and B4) were extracted 1 time followed by 3 successive rinsing steps. 5 mL ammonia/ammonium chloride buffer were added to each sample. The samples were vigorously shaken and ultrasonicated with 100 mL ethyl acetate/methanol mixture (1:4 v/v) for 30 min. The organic phases were transferred into round bottom flasks using folded filter papers (MN615 ¼. ∅150 mm; Macherey-Nagel, Germany) and the extraction procedure was followed by rinsing 3 times, each one with 50 mL for 15 min.

The combined extracts were filtered and both collecting flasks and filter papers with sodium sulfate were rinsed with 25 mL of the extracting solvent. At this step, B1 and B2 were spiked. Thereafter, raw extracts were rotary evaporated nearly to dryness. The residue were reconstituted in 10 mL methanol and filtered using 0.2-µm polyester syringe filter and then finally cleaned-up. After the clean-up procedures, B3 and B4 were spiked. Finally, 7 µL of these solutions were injected into LC/MS/MS. The efficiency of proposed extraction procedure was tested at different spiking levels, e g., 4, 50 and 100 µg/kg fresh manure.

Number of extraction cycles required to achieve exhaustive extraction procedure was tested. Six liquid manure samples, each one contained 2.5 g dry substance, were filled in 300-mL Erlenmeyer flasks. Four samples were spiked with the target compounds at 100 µg/kg fresh manure, whereas the other two were left unspiked. All samples were frozen at -20 °C for 8 h and lyophilized at -50 °C for 24 h. Two spiked samples were vigorously shaken with 100 mL ethyl acetate and methanol mixture (4:1, v/v) for 2 min and ultrasonicated for 15 min. The organic phases were transferred into round bottom flasks, rotary evaporated and cleaned-up. Finally, 7 µL were injected into LC/MS/MS.

The extraction procedure was followed by 5 rinsing steps, where the already extracted samples were ultrasonicated with 50 mL solvent mixture for 15 min. The obtained organic phases were separately kept after every step, rotary evaporated and cleaned-up. Finally, 7 µL were injected into LC/MS/MS. Only organic phases of step 5 and 6, subsequently, they were combined before analysis. The other two spiked and two unspiked samples were extracted in 4 steps, first was ultrasonication with 100 mL of solvent mixture for 15 min and then 3 rinsing steps via ultrasonication with 50 mL for 15 min. The organic phases of the 4 steps of each sample were combined rotary evaporated, cleaned-up and Finally, LC/MS/MS analyzed. The unspiked samples were spiked before the clean-up procedure with the target compounds at the same concentration. Number of extraction cycles was established by comparing the recovery of each extraction cycle and recovery obtained by 4 extraction cycles or the recovery of zero samples spiked after extraction but before the clean-up procedure.

3.4.3 Soil and manured soil samples

3.4.3.1 Direct solvent extraction

Soil samples

To efficiently extract the target compounds from soil samples, several series of fortification experiments were carried out using 2 differently textured soil samples, i.e., sand and clay soil. Extraction procedures were conducted using different solvents. Four replicates, each one contained 50 g soil samples, were spiked with the target compounds at 100 µg/kg fresh soil. Soil samples were mixed well using a spatula and left for 1 h until the solvent was evaporated. 5 mL ammonia/ammonium chloride buffer were added to the spiked samples directly before extraction. The samples were shaken with 100 mL of the extracting solvent (or solvent combination) in 300-mL Erlenmeyer flasks using a horizontal shaker (Type 3020; Gesellschaft für Labortechnik, Burgwedel, Germany) at 250 rpm overnight. The organic phases were transferred into round bottom flasks using folded filter papers (Macherey-Nagel, Düren, Germany). These samples were rinsed 2 additional times with 100 mL of the solvents. The shaking times were, 2 h and 1 h, respectively. The combined extracts were filtered using the same folded filter papers with higher amounts of anhydrous sodium sulfate. For minimizing the losses during this procedure, both collecting flasks and filter papers with sodium sulfate were rinsed with 25 mL of extracting solvents. The extracts were rotary evaporated nearly to dryness and the remaining solvents were further evaporated under a gentle nitrogen stream. The extract residues were re-dissolved in 10 mL methanol and microfiltered through 0.2-µm polyester filter into 10 mL volumetric flasks. The raw extracts were cleaned-up using SEC and SPE and finally 7 µL were injected into LC/MS/MS.

For the solvent selection, different solvents and some of their combinations including methanol, acetonitrile, acetone, ethyl acetate, methanol/ethyl acetate mixture at ratios of 1:1 or 1:4 (v/v) and an acetone/ethyl acetate mixture (1:4 v/v) were tested at original pH values of the samples. The effect of pH on the extraction of target compounds was tested as well. The efficiency of ethyl acetate, acetone and ethyl acetate (1:4 v/v) and methanol/ethyl acetate mixture (1:4 v/v) to extract the target compounds at alkaline condition were checked. pH adjustment was performed by adding 35 mL ammonia/ammonium chloride buffer using ethyl acetate or 5 mL in case of the solvent mixtures. Another series was extracted at acidic conditions using 0.5 mL formic acid and the latter solvent mixture.

For solvent selection in case of clay soil, methanol, acetone, ethyl acetate were tested for analytes extraction at original pH. The efficiency of methanol/ethyl acetate mixture (1:4 v/v) to extract the target compounds spiked at 2, 4, 40 and 100 µg/kg for sand soil and at 4, 40, and 100 µg/kg for clay soil were investigated at alkaline pH.

Manured soil samples

Four replicates, each one contains 10 g pig excrement (11.1 % ds), were weighed in 300-mL Erlenmeyer flasks. They were spiked with the target compounds at 100 µg/kg and manually shaken for 30 s. Then, 50 g sieved (2 mm) soil samples were added and mixed well using a spatula. 5 mL ammonia /ammonium chloride buffer were added.

These samples were shaken with 100 mL methanol/ethyl acetate mixture (1:4) overnight. After removal of the organic phases using folded filter papers, the extracted samples were rinsed twice with 100 mL of solvent mixture by shaking at 250 rpm for 2 h and 1 h, respectively. These samples were rinsed again 2 additional times with 100 mL of solvent mixture for 2 h and 1 h, respectively, to test the exhaustive power of the extraction procedure. Based on these 5 steps, the number of extraction cycles which are required for efficient extraction was defined.

The combined extracts were filtered using folded filter papers with anhydrous sodium sulfate. Flasks and filter papers with sodium sulfate were rinsed with 25 mL of the solvent. The extracts were rotary evaporated nearly to dryness and the remaining solvents were further evaporated under a gentle nitrogen stream. The obtained residues were re-dissolved in 10 mL methanol and filtered through 0.2-µm polyester filters into 10-mL volumetric flasks. The raw extracts were cleaned-up using SEC and SPE. 7 µL were injected into LC/MS/MS. Efficiency of the extraction procedure was tested using different spiking levels of the analytes. The analytes were spiked into manured sand soil at 2, 10, 50, and 100 µg/kg and at 2 and 100 µg/kg into manured clay soil. The spiked concentration of the chloro-analogue of flubendazole used as the internal standard was 8 µg/kg.

3.4.3.2 Ultrasound-assisted extraction

The second extraction technique was checked only for manures soil samples. Four replicates, each containing 10 g of homogenized manure sample, were weighed in 300-mL Erlenmeyer flasks. They were spiked with the target compounds at 100 µg/kg and manually shaken for 30 s. Then, 50 g of sieved (2 mm) soil samples were added and mixed well using a spatula. The spiked samples were buffered by adding 5 mL of ammonia/ammonium chloride buffer. Manured soil samples were mixed again using a spatula. After standing for 1 h, 100 mL methanol/ethyl acetate mixture (1:4) were added to the buffered samples and then sonicated for 30 min. The extracted samples were rinsed two times with 100 mL of the solvent mixture for 15 min. The extracts from all cycles for a given sample were combined, rotary evaporated, cleaned up and finally measured using LC/MS/MS. The efficiency of the extraction procedure was investigated at different spiking levels. The spiking concentrations

were 2, 10, and 100 µg/kg for manured sand soil, while 2 and 50 µg/kg for manured clay soil. The spiking concentration of the internal standard was 8 µg/kg.

3.5 Clean-up procedures

The clean-up procedure for the raw extracts is a critical step with relevant impacts on the subsequent analytical process, especially when LC/MS/MS with electrospray ionization (ESI) is applied for analysis of environmental samples of high complexity and variability. The matrix components in manure, soil and manured soil samples can cause unpredicted effects on the responses of target compounds. Therefore, sophisticated clean-up procedures are needed to enhance the analytical quality. Two steps were used to clean-up the raw extracts, i.e., size exclusion chromatography (SEC) followed by solid phase extraction (SPE). Additionally, the efficiency of n-hexane for lipid removal from manure samples was also tested.

3.5.1 Size exclusion chromatography

Size exclusion chromatography (SEC) was applied in order to remove co-extractants of higher molecular weight substances such as humic and fulvic acids from liquid manure and soil samples. For this purpose, the Gilson GPC unit was used. This system consisted of isocratic medium pressure pump model 302, fraction collector model 201 (Gilson, Düsseldorf, Germany) and glass column filled with Sephadex (58 cm length, 25.4 cm i.d.; Latek, Eppelheim, Germany). 5 mL of micro-filtered methanolic raw extracts were manually injected using a 10-mL syringe (Hamilton, Reno, NV, USA). First, methanol acidified with 0.01 M acetic acid was used as a mobile phase with a flow rate 5 mL/min. The injection volume was 5 mL. The fractions were collected at 25 min for the waste fraction, 50 min for the analyte fraction and finally 13.5 min for rinsing. The analytes fractions (225 mL) were rotary evaporated nearly to dryness. Additionally, pure methanol was also tested as the mobile phase.

3.5.2 Solid phase extraction

For additional clean-up, solid phase extraction (SPE) was applied using two different cartridges, e.g., SDB1 (200 mg, 6 mL; Mallinckrodt Baker, Griesheim, Germany) and Oasis HLB (60 mg, 3 mL, Water, Millford, MA, USA). 5 mL of the extracts obtained from the first clean-up step, were diluted with 200 mL deionized water. The SPE cartridges were conditioned

prior to sample extraction with 5 mL methanol and equilibrated with 10 mL of acidified deionized water. The diluted extracts (200 mL) were adjusted to pH 2.3 with formic acid, ultrasonicated for 1 min, filtered with 0.6-µm glass fiber filters and then finally enriched in the cartridges. The samples were passed through the SPE cartridges at a flow rate of 4 mL/min. The cartridges were washed by 5 mL deionized water and dried under low pressure for 10-15 min. The dried cartridges were eluted with 2 x 5 mL methanol. The eluates were evaporated nearly to dryness under a gentle nitrogen stream and reconstituted to the final volume of methanol. 1-mL aliquots of these eluates were transferred to 1.5-mL autosampler vials for LC/MS/MS analysis. Different eluents were tested for the analyte elution step, e.g., acetonitrile and ethyl acetate. Furthermore, the effects of the filtration process using 0.6-µm glass fiber filter and addition of NaCl (5 g) during this step were tested as well.

3.5.3 Lipid removal using n-hexane

In several studies, n-hexane was described as efficient methods to remove the lipids from, e.g., manure samples (Pfeifer et al., 2002; Su et al., 2004). Thus, the efficiency of n-hexane to remove the lipid and its effect on the recovery of the target compounds was tested in two series. First, SPE cartridges were washed with 10 mL n-hexane prior to the elution step. Second, lyophilized samples were rinsed by shaking with 100 mL n-hexane. For this purpose, 4 lyophilized manure samples were prepared. Two spiked samples with the target compounds at 100 µg/kg fresh manure (S1 and S2) and two unspiked (B2 and B2), were treated with 100 mL n-hexane. The samples were shaken on a horizontal shaker at 220 rpm for 5 h. The n-hexane phases were decanted, rotary evaporated, cleaned-up and finally analyzed using LC/MS/MS. Thereafter, the solid parts were extracted by methanol as described in detail in section 3.4.2.2. The raw extracts of unspiked samples were spiked with target compounds at the same concentration. Finally, extracts were cleaned by SEC. The mean recoveries of the samples spiked before rinsing were then compared with the recovery of the samples spiked after extraction but before clean-up procedure.

3.6 High-performance liquid chromatography with variable wavelength detection

3.6.1 Theoretical aspects

High-performance liquid chromatography (HPLC) is used to separate mixtures of organic compounds into their individual components based on partitioning between two phases re-

ferred to mobile phase and stationary phase. HPLC is suitable for analysis of micro and macro molecules, thermally labile, thermally stable and hydrophilic compounds mainly without any need for derivatization. Since, the pharmaceutical compounds have been considered as a new group of contaminants, HPLC has become the backbone of environmental analysis. Thus, reversed phase (RP) HPLC, defined by a non-polar column in combination with polar solvents, was used to analyze two polar compounds and eight of their corresponding highly polar metabolites. Different detectors are coupled with HPLC instruments, e.g., variable wavelength (UVD), and diode array (DAD) and mass selective detectors.

3.6.2 Operational conditions

Preliminary, HPLC/UVD and HPLC/DAD were applied in order to study the chromatographic behavior of the compounds under study. For this purpose, all HPLC runs in preliminary experiments were carried out using an HPLC system with column oven, auto-sampler at ambient temperature, binary pump and a variable wavelength detector (UVD) (HP 1050 Series; Agilent, Waldbronn, Germany). Additionally, an HPLC system, Agilent 1100 series HPLC-system (Agilent Technologies, Waldbronn, Germany) equipped with a binary pump, column thermostat, thermostated autosampler, vacuum degasser system and photodiode-array (DAD) detector controlled by an HP ChemStation was also used to obtain the absorption spectra for target compounds with wavelength ranging from λ = 200 to 400 nm. Chromatographic separation was performed on the reversed phase column Eclipse XDB (150 mm x 4.6 mm, 5 µm; Agilent Technologies, Santa Clara CA, USA). Eluents were consisted of two mobile phases. Phase (A) consisted of 0.5 % formic acid in water, while phase B consisted of 0.5 % of formic acid in water and acetonitrile (25:75 v/v). The flow rate was 1 mL /min and the injection volume was 10 µL. The gradient started at 10 % B, increased to 24 % by 25 min, 35 % by 45 min, 100 % by 50 min and then decreased to 10 % by 52 min. The column re-equilibrated for 5 min before the next injection. The HPLC system was operated at different wavelengths. Finally, λ = 280 nm was selected. All compounds were dissolved in methanol and separated in one single gradient run.

3.7 High-performance liquid chromatography with tandem mass spectrometry

3.7.1 Theoretical aspects

High-performance liquid chromatography coupled with mass spectrometer (LC/MS/MS) is a powerful tool that can be used to quantify and to verify the identity of chemical compounds even at low concentration in complex and heterogeneous sample matrices. In addition to sample introduction systems such as HLPC, ion source, mass analyzer and detector are the main components of the mass spectrometry as illustrated in **Figure 3.1**.

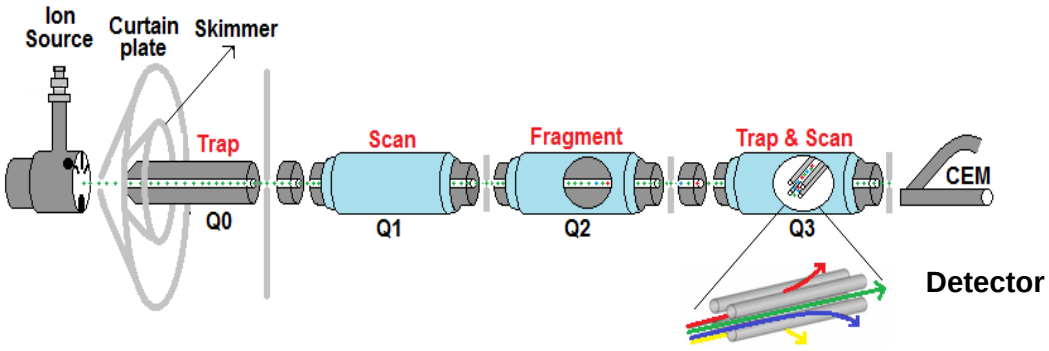


Figure 3.1: Assembly of a triple stage quadrupole mass spectrometer where the linear ion trap mass spectrometer was created using either q2 or Q3 in 4000 QTRAP (according to Hager, 2002; Müller, 2004 and AB Sciex, 2009).

3.7.1.1 Ion sources

The ion source is one of the most critical part of the mass spectrometer. Three ion sources, e.g., electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization sources (APPI) are available and widely used in the LC/MS/MS systems. The selection of the used ion source is depending on the physicochemical properties of the analytes and matrices under study.

For analytes of intermediate to high polarity and molecular weight, ions are produced under atmospheric pressure (API) using ESI or APCI. ESI is the softest and most usual ionization technique. In ESI, solvent containing analytes are forced through a capillary needle at optimal flow rate. The needle is subjected to a high voltage in positive or negative mode based on the chemical characteristics of the compounds under study. The droplets containing analytes are produced at the tip of the capillary and assisted by the nebulizing gas. Based on the polarity of the voltage applied onto the needle, positive or negative charged droplets are produced. During liquid phase ionization, these charged droplets are subjected to successive

evaporation. The ionization is supported by the heated drying gas. Due to solvent evaporation, these droplets are shrinking to give fine droplets containing higher numbers of charged molecules. When the charge density at the surface of the droplets reached the Rayleigh limit, an electric repulsion force, namely columb explosion, can overcome the surface tension force of the very fine droplets. This process is repeated until the molecular ions are released and enter the mass analyzer. At this time, the curtain gas that flows between the orifice and the curtain plate prevents the solvent droplets from entering the quadrupole system in order to keep it clean (**Figure 3.2**). Positive ions are formed, such as precursor ion $[M+H]^+$ and $[M+Na]^+$ adducts when a positive voltage is applied onto the capillary. In the negative-ion mode, $[M-H]^-$ is the mainly produced ion (Griffiths et al., 2001; Stroobant and Hoffmann, 2007).

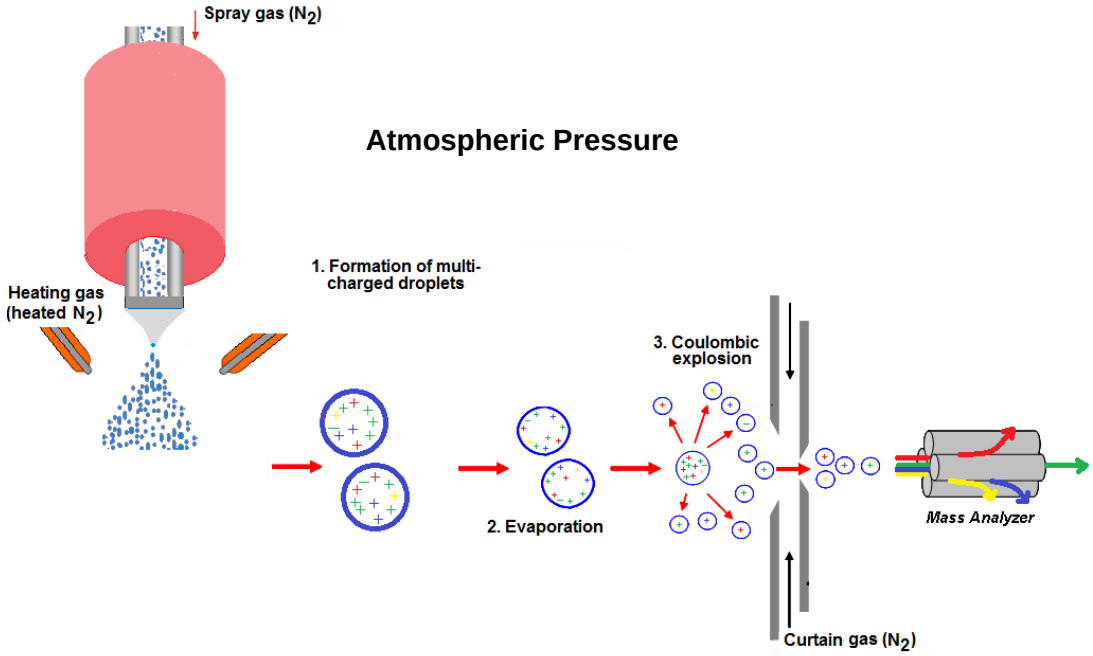
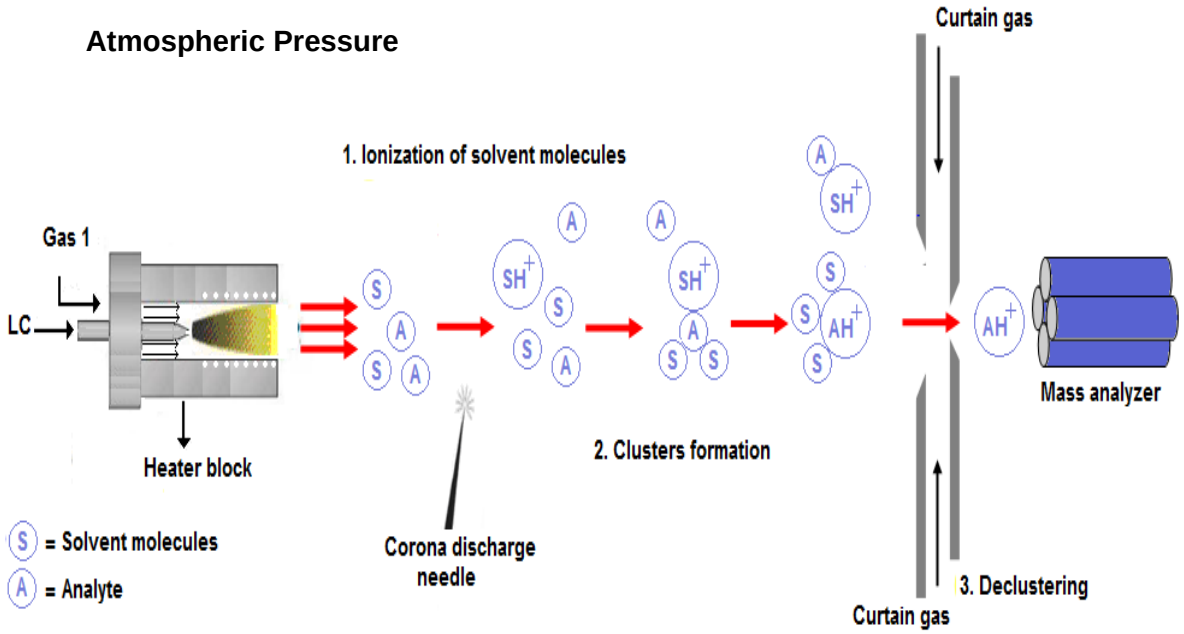


Figure 3.2: Ion source and ion formation in electrospray ionization (ESI) (according to Griffiths, 2001; Müller, 2004 and AB Sciex, 2009).

In contrast to ESI, APCI is suitable for small, relatively non polar and thermostable molecules. Liquid samples, introduced via HPLC, pass through a heated needle causing a dispersion of these samples. No voltage is applied onto the capillary. Vaporizing heater blocks around the needle assists to evaporate the mobile phase containing analytes in the heated chamber at 400 °C. Drying gas flow combined with the heat of heater blocks, complete the vaporization process and produce clouds of solvent and intact analyte molecules. The ionization process starts when the gas molecules of nitrogen and oxygen are exposed to the corona needle, where a current up to 5 µA is applied creating a stream of electrons that serve to

ionize the solvent of the mobile phase. Charges are transferred to the solvent molecules, e.g., water, methanol or acetonitrile, and then finally to analyte molecules in the gas phase. This gas phase ionization is described in the next equations. The protonated species, e.g., $CH_3OH_2^+$ and H_3O^+ which are present in the vapor state transfer protons to the analytes that are present in the vaporized state according to their proton affinity (Edward and Henion, 1998). **Figure 3.3** shows the principle of the ionization process using an APCI source.



$$N_2 + e^- \rightarrow N_2^{+\cdot} + 2e^-$$

$$N_2^{+\cdot} + H_2O \rightarrow N_2 + H_2O^{+\cdot}$$

$$H_2O + H_2O^{+\cdot} \rightarrow H_3O^+ + HO^{\cdot}$$

$$M + H_3O^+ \rightarrow [M+H]^+ + H_2O$$

Figure 3.3: Ion formation mechanisms in atmospheric pressure chemical ionization (APCI) (according to Edward and Henion, 1998; Müller, 2004 and AB Sciex, 2009).

Based on the chemical properties of the analyte, sensitivity of the analyte signal and application (matrix), one of the above ion sources can be selected. **Table 3.3** shows a comparison between ESI and APCI.

Table 3.3: Characteristics of electrospray (ESI) and atmospheric pressure chemical ionization (APCI) (Bruins, 1994; Thurman et al., 2001; Liang et al., 2003; Mallet et al., 2004; Souverain et al., 2004).

	ESI	APCI
Similarities	- ionization under atmospheric pressure. - softest ionization mode.	- ionization under atmospheric pressure. - soft ionization mode.
Differences	- suitable for low and high masses, polar and highly polar and thermolabile analytes. - liquid phase ionization. - compatible with flow rates from 5 µL to 2 mL.	- suitable for small masses, volatile, polar, relatively non polar and thermostable analytes. - gas phase ionization. - compatible with flow rates from 200 µL to 2 mL.
Disadvantages Matrix effect	- signal suppression or enhancement.	- slight effects.
Adduct and cluster formation	- adducts could be formed, e g., $[M+NH_4]^+$, $[M+Na]^+$ and $[M+K]^+$.	- clusters or dimmers could be formed, e. g., $[M+CH_3OHH]^+$, $[M+CH_3CNH]^+$ and $[M+M+H]^+$.

3.7.1.2 Mass analyzer

Triple quadrupole (QqQ) and 3D-ion trap (IT) systems are the most frequently used mass analyzers. The first one is preferably applied for target compound analysis operating in multiple reaction monitoring mode (MRM). Due to its high selectivity corresponding to high sensitivity, VMP in complex sample matrices at low µg/kg concentrations can be determined. Due to the opportunity of carrying out MS^n experiments, the 3D-ion trap is preferably applied for screening analysis of unknown analytes. Both advantages are combined with the 4000QTRAP instrument (AB Sciex, Darmstadt, Germany) applicable as a triple stage quadrupole instrument, additionally equipped with linear ion trap. The quadrupole mass spectrometer consists of an ion source (ESI and/or APCI) and a mass filter consisting of four parallel metal rods arranged as in the **Figure 3.2**. Each two opposite rods have an applied direct-current of positive charge and the other two are connected to the negative end. Due to this hybrid design, several scans can be achieved, some are available in the triple quadru-

pole mode and the others are available in the ion trap mode. In the triple quadrupole mass analyzer both Q1 and Q3 are considered as mass filters, while q2 is the collision cell for fragmentation of precursor ions to form the corresponding product ions.

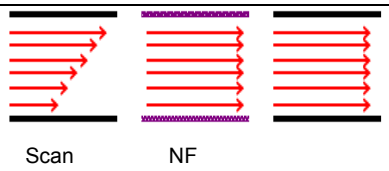
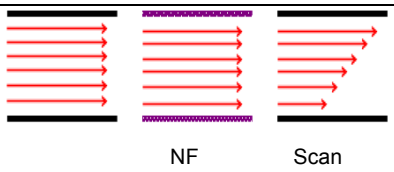
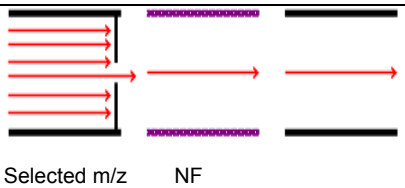
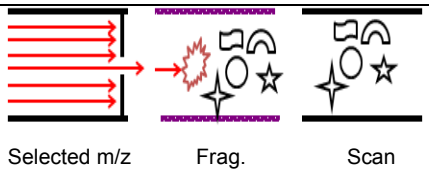
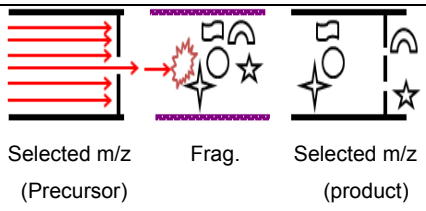
Precursor ions are separated by the mass analyzer (Q) according to mass (m) per charge (z) ratio (m/z). This ratio is usually equal m because z equals 1 at low mass range < 1000 Da (Stroobant and Hoffmann, 2007). A combination of direct current (DC) and radio frequency (RF) voltages are applied onto the mass filter and allows the entire mass range to be scanned in full scan modes by first mass analyzer (Q1) in the triple quadrupole instrument or by the second mass analyzer (Q3). Moreover, Q1 scan can be fixed to select one precursor ion as in multiple ion scan.

The mass analyzer can be fixed at specific masses, e.g., product ion scans (PI) in which the Q1 is fixed at specific precursor ions. The isolated precursor ion collides with target gas in q2 at specific collision energy producing fragments. After fragmentation, the Q3 allows transmission of all product ions based on m/z. Finally, product ion spectra are recorded. Mass spectra are the outcomes where mass intensity is plotted versus m/z ratio. The ions which are selected by Q1 (MS scan) are referred as the parent or precursor ions. The ions which are produced in q2 and selected by Q3 (MS/MS) are referred as the daughter or product ions.

Multiple reaction monitoring (MRM) methods are based on the output of the two aforementioned scans. Specific precursor ion or ions formed in ion source (ESI or APCI) are selected by Q1 under control of the applied DC and RF. At specific values of the DC and RF, one mass is stable and successfully travels through Q1 and the others are unstable and they will be lost (March, 1997) (**Figure 3.4**). The selected ions are accelerated into the linear accelerating collision cell (LINAC, q2), where they collide with neutral gas (nitrogen) to produce characteristic fragments (PI) based on applied collision energy (CE) and pressure of collision activated dissociation gas (CAD). Further selection for specific fragments is achieved by Q3. The more specific ions with highest intensities are usually used to build efficient MRM methods.

More information for structural characterization could be obtained when Q3 is operated as ion trap, where the ions are trapped and stored to increase the sensitivity as present in enhanced mass scan (EMS) and enhanced product ion scan (EPI) or to increase selectivity by MS^3 scan by production highly specific granddaughters ions. **Table 3.4** represents the used scan types in triple quadrupole mode within this method development.

Table 3.4: Applied scan types in the method development using the 4000QTRAP LC/MS/MS system (according to Müller, 2004; Stroobant and Hoffmann, 2007 and AB Sciex, 2009).

Scan type and its application	Q1 q2 Q3
Full scan (Q1 or MS): Used to check the presence of precursor ions of target compounds as the first step to build MRM for quantitation purposes.	
Full scan (Q3 or MS): Used to check the presence of precursor ions of target compounds for quantitation purposes.	
Multiple ion scan: Used to check the presence of precursor ions of the target compounds during manual compound optimization for qualitative purposes.	
Product ions scan (PI): Full scan for all the fragments of selected compounds. It is considered as second step to build MRM for quantitation purposes.	
Multiple reaction monitoring (MRM): Precursor ions are selected by Q1, fragmented in q2 and then specific fragments are selected by Q3 for the identification and quantitation of the target compounds	

Frag. = Fragmentation, NF= No fragmentation

EPI is considered as full scan mode and used to detect all the fragments of selected compounds but with higher sensitivity than normal PI. This is based on fill time when Q3 is operated in the trap mode. The latter was combined as dependent scan with MRM scan as full scan to confirm the positive results using Information Dependent Acquisition (IDA) option, where in the 4000QTRAP IDA method enables the mass spectrometer to switch between different modes within one run.

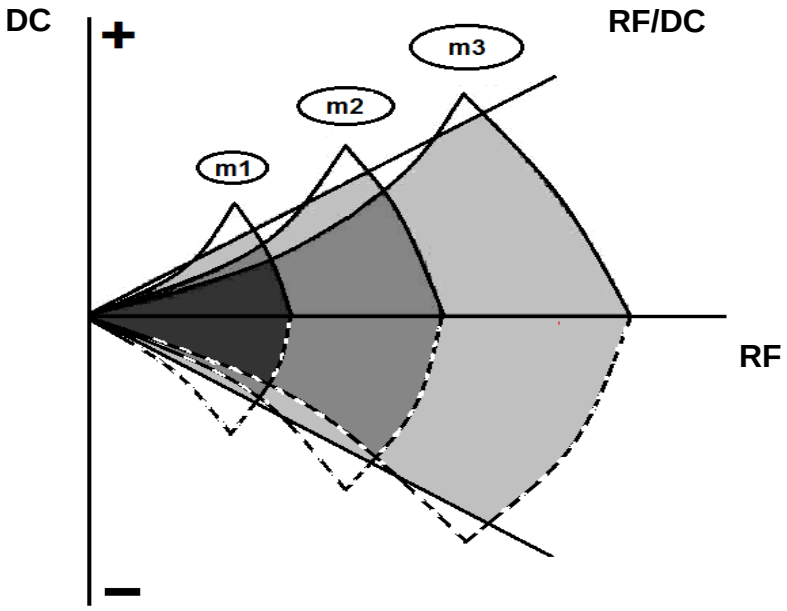


Figure 3.4: Mathieu stabilty diagram where each quadrupole acts as mass filter based on constant ratio of direct current (DC) and radio frequency (RF) (according to March and TODD, 2005; Stroobant and Hoffmann, 2007).

3.7.1.3 Detectors

In mass spectrometry, the secondary electron multiplier (SEM) is the most frequently used ion detector today (March and TODD, 2005; Stroobant and Hoffmann, 2007). SEM usually consists of a sequence of 12 to 20 dynodes held at decreasing negative potentials through a sequence of resistors. It is usually made from copper/beryllium alloy which has a high efficiency to emit secondary electrons. When the detected compounds are in the positive mode, a high voltage from -3 to -30 kV is applied on the first dynode, i.e., the conversion dynode, whereas the output of the multiplier remains at ground potential. Each dynode is held at a lower negative potential than the previous one to accelerate the ion movement. After exit, the ions from the mass analyzer, the energetic ions strike the surface of conversion dynode, where several secondary electrons are released. These secondary electrons are reflected to opposite direction to hit the inner surface of the next dynode causing the emission of more electrons. This process is repeated several times to cause an amplification of resulting electrons. Finally, this continuous flow of the electron current is amplified to certain factor equal to the number of present dynodes. **Figure 3.5** shows the principle of the ion detection by an electron multiplier detector.

A horn-shaped channel electron multiplier (CEM) is the common version of the SEM used in 4000 QTRAP mass spectrometers. The latter is more compact and produce more sensitivity

than the others equal ≥ 10^8 because it consisted of continuous dynodes instead of separate dynode in a normal SEM.

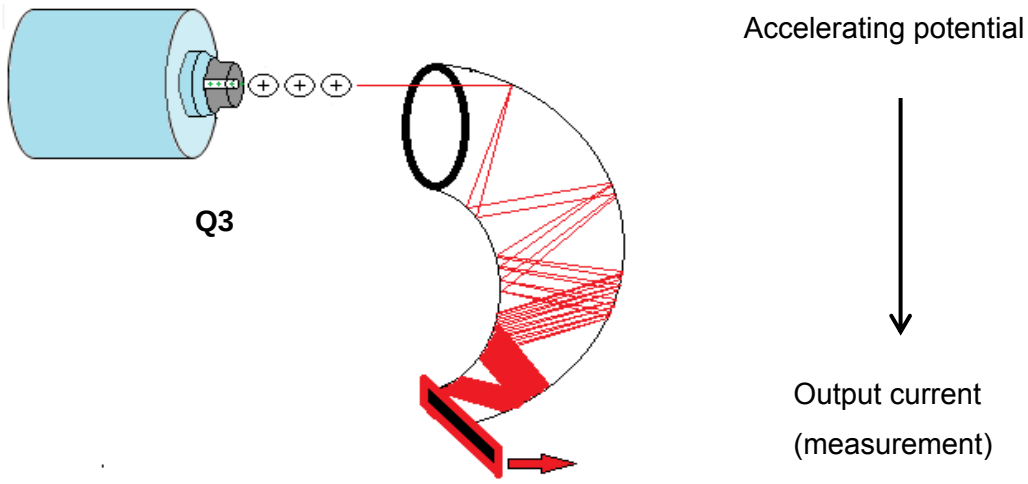


Figure 3.5: Principle of ion detection by an electron multiplier detector (according to Stroobant and Hoffmann, 2007).

3.7.2 Operational conditions

3.7.2.1 Instrument properties

Based on the chromatographic conditions of HPLC/UVD experiments, the LC//MS/MS method was developed for water analysis. The flow rate was again 1 mL/min and the injection volume was 10 µL using the same gradient and the mobile phases as mentioned in section 3.6.2. Within the method advancement, a new gradient was established by mixing another two mobile phases. Mobile phase A consisted of 10 % acetonitrile in water (v/v) with 0.01% formic acid while phase B consisted of pure acetonitrile. The gradient started at 10 % B, increased to 24 % by 25 min, increased to 100 % by 35 min and then kept isocratic with 100 % B by 40 min. Finally, B decreased to 10 % by 42.5 min and kept isocratic with 10 % B by 47.5 min. The stop time was 47.5 min. The flow rate was 1 mL/min. 7 µL were used during analysis of the target compounds in the matrices under study. Using these chromatographic conditions, different parameters, e.g., injection volumes, different temperatures and flow rates were tested. The column was rinsed every day after the sample measurement at least 30 minutes with acetonitrile. Instead of acetonitrile, methanol was tested for target compound separation under the mentioned chromatographic conditions. The separation conditions using LC/MS/MS are given in **Table 3.5**.

Table 3.5: Chromatographic conditions used in LC/MS/MS methods.

Column	C18 Zorbax Eclipse endcapped XDB column (150 mm x 4.6 mm, 5µm).
Eluent 1	A: 0.01% of formic acid in a mixture of water and methanol (90:10, v/v). B: methanol.
Eluent 2	A: 0.01% of formic acid in a mixture of water and acetonitrile (90:10, v/v). B: acetonitrile.
Gradient	Step / Time / Eluent A [%[1 / 0.0 / 90 2 / 25.0 / 76 3 / 35.0 / 00 4 / 40.0 / 00 5 / 42.5 / 90 6 / 47.5 / 90
Flow rate	1 mL/min
Column temperature	23 °C
Injection volume	7 µL

LC/MS/MS analysis was performed using a system consisted of an Agilent 1200 SL Series liquid chromatography including a vacuum degasser, a binary pump SL, and high performance auto-sampler SL (Agilent Technologies, Waldbronn, Germany). This sample introducing system coupled to an AB SCIEX 4000 QTRAP tandem mass spectrometer (Darmstadt, Germany) equipped with an electrospray ionization interface (ESI; Turbo V^{TM} source). The LC/MS/MS system was controlled and data were evaluated by Analyst software (version 1.5).

3.7.2.2 Method optimization

To create MRM methods for LC/MS/MS analysis, several parameters had to be optimized. These parameters classified into two categories. Parameters which are depending on chemical properties of the analytes and defined as compound dependent parameters. Ion source parameters are depending on the flow rate in the chromatographic method. The compound

and ion source parameters were optimized by infusion and flow injection analysis (FIA-MS/MS), respectively.

The main compound dependent parameters, i.e., entrance potential (EP), collision cell exit potential (CXP), declustering potential (DP), collision energy (CE) and collision gas (CAD) have to be checked by infusion optimization. For this purpose, the individual solutions of target compounds were continuously infused into the interface of the ion trap tandem mass spectrometer. Methanolic solutions with different concentrations according to the sensitivity of the target compounds were used. These solutions were introduced into the ion source via an integrated syringe pump (Harvard Apparatus 22, South Natick, MA, USA). The flow rate was 10 and 20 µL/min for ESI and APCI, respectively. The concentrations of the standard solution ranged from 10 to 100 ng/mL. Finally, initial MRM methods containing compound dependent parameters were individually created for each compound. All of these methods for different compounds were merged in one method to optimize ion source parameters. Precursor ions, MRM transitions for quantifier and qualifier ions and selected setting parameters including declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) in positive and negative ionization modes are listed in **Table 3.6** and **3.7**.

Flow injection analysis (FIA) was applied to optimize instrument parameters. For this purpose, methanolic standard solutions of target compounds were mixed with the mentioned mobile phase A/B at a 65:35 ratio through the peak for tuning, using the pump, autosampler and the mass spectrometer. The system operated in isocratic mode to optimize the ion source parameters. The flow rate was 1 mL/min and injection volume was 10 µL.

First, ion source parameters including, curtain gas (CUR), source temperature, nebulizing gas (GS1), desolvation gas (GS2), collision gas (CAD), ion source voltage (IS) and interface (Ihe) were optimized using the initial MRM method and ESI interface. The same parameters were optimized as well as needle current (NC) using initial MRM method and APCI interface. The values of ion source operational parameters using ESI and APCI interfaces are listed in **Table 3.8**.

Q1 scan was used to acquire and record precursor ion mass spectra for each compound individually using ESI and APCI in positive and negative ionization mode. Product ions mass spectra were also recorded in negative and positive ion mode.

Based on the two above mentioned scans where the base peak ions were selected as quantifier ions and the next ones with highest intensities were selected as qualifier ions. Thus, the individual MRM methods were built. Finally, all of these MRM methods were merged in final multi-component MRM method. Using the same chromatographic conditions, all compounds were measured in positive and negative ion modes using LC/MS/MS/MRM with ESI. The response of target compounds using APCI was also evaluated.

Table 3.6: MS/MS parameters of positive ion mode using electrospray (ESI) and atmospheric pressure chemical ionization (APCI) interfaces.

Analytes	MW [g mol^{-1}]	$[M+H]^+$ [amu]	Ion 1 [amu]	Ion 2 [amu]	DP	CE		EP	CXP	
						Ion 1	Ion 2		Ion 1	Ion 2
FEN	299.0	300.0	268.0	159.0	91	37	51	10	18	10
FEN-SO	315.2	316.2	159.0	191.1	86	51	33	10	10	12
FEN-OSO	331.2	332.2	300.2	159.2	96	33	53	10	20	10
FLU	313.0	314.0	281.8	95.2	86	33	73	10	6	16
FLU-M1	315.3	316.3	284.0	97.2	96	33	57	10	6	6
FLU-M2	255.2	256.2	123.0	95.0	101	61	61	10	4	4
FLU-M3	257.2	258.2	240.3	134.2	111	41	39	10	16	8
FLU-M4	271.2	272.2	148.2	97.2	116	39	47	10	8	6
FLU-M5	240.2	241.2	123.0	95.0	81	61	61	10	18	18
FLU-M6	256.2	257.2	123.3	95.0	86	33	43	10	6	16
IS	329.1	330.1	298.0	139.0	86	31	53	10	20	8

MW = Molecular weight, amu = Atomic mass unit, DP = Declustering potential, CE = Collision energy, CXP = collision cell exit potential, EP = Entrance potential

Table 3.7: MS/MS parameters of negative ion mode using electrospray (ESI) and atmospheric pressure chemical ionization (APCI) interfaces.

Analytes	MW [g mol^{-1}]	$[M+H]^-$ [amu]	Ion 1 [amu]	Ion 2 [amu]	DP	CE		EP	CXP	
						Ion 1	Ion 2		Ion 1	Ion 2
FEN	299.0	298.0	266.1	189.0	-85	-26	-44	-10	-15	-15
FEN-SO	315.2	314.0	205.0	157.0	-80	-36	-64	-10	-11	-11
FEN-OSO	331.2	330.0	298.1	157.1	-140	-34	-56	-10	-5	-1
FLU	313.0	312.0	280.0	157.0	-26	-60	-45	-10	-7	-11
FLU-M1	315.3	314.2	282.0	264.0	-80	-48	-100	-10	-7	-9
FLU-M2	255.2	254.0	131.0	115.0	-20	-56	-66	-10	-9	-7
FLU-M3	257.2	256.0	238.0	222.0	-95	-26	-38	-10	-3	-11
FLU-M4	271.2	270.0	238.0	236.0	-100	-34	-46	-10	-5	-45
FLU-M5	240.2	239.2	116.0	115.0	-125	-48	-68	-10	-7	-5
FLU-M6	256.2	255.0	132.0	89.0	-60	-48	-76	-10	-9	-13

MW = Molecular weight, amu = Atomic mass unit, DP = Declustering potential, CE = Collision energy,

CXP = collision cell exit potential, EP = Entrance potential.

Table 3.8: Ion source parameters using electrospray (ESI) and atmospheric pressure chemical **ionization** (APCI).

Ion source parameters	ESI	APCI
Curtain gas (CUR)	20.7 x 10^4 Pa	20.7 x 10^4 Pa
Ion source temperature	600 °C	400 °C
Nebulizing gas (GS1)	55.0 x 10^4 Pa	14.0 x 10^4
Desolvation gas (GS2)	41.2 x 10^4 Pa	41.2 x 10^4 Pa
Collision gas (CAD)	medium pressure	medium pressure
Ion source voltage (IS) Needle current (NC)	4 kV ----	---- 3 µA
Interface (Ihe)	on	on

Triple quadrupole mode (QqQ) using MRM scan of 4000 QTRAP instrument was used for identification and quantitation purposes. Furthermore, the linear ion trap was applied for enhanced product ion scans for identification and confirmation. Using information dependent acquisition (IDA) in 4000 QTRAP instrument complementary data are simultaneously obtained to give maximum structural information with a minimum number of analytical runs and decreasing the cost of the analyses. In IDA method, MRM and enhanced product ion scans (EPI) are combined. When MRM ions are detected with intensity more than 5000 counts per second (cps), this threshold value was operationally defined, IDA method switches the instrument from survey scan using MRM mode to EPI scan and triggers the second experiment. At this time, Q3 in the linear ion trap mode starts to collect the analyte product ions for a certain time (40 ms) or using dynamic fill time (DFT) which was automatically calculated. The scan rate was 1000 cps and collision energy spread (CES) was set at 15 V. Afterwards, qualified, full enhanced product ion spectra were obtained parallel with the MRM chromatograms. Based on the latter scan, the comparison between not only the MRM ions but also all the fragments obtained in enhanced product ions spectra using reference standard solutions of the target compounds and those produced in real samples can be achieved. The mass spectrometer operated with unit resolution for both mass analyzers and the compounds were measured with a dwell time of 150 ms for each.

3.8 Method validation

3.8.1 Principal aspects

Validation is one critical process achieved by testing different parameters to confirm that the purposed analytical method conforms to defined use requirements and will be within specification under defined operational conditions (Careri and Mangia, 2006).
The main objective of this process is fitness for purpose which is defined by IUPAC (2002) as "degree to which data produced by a measurement process enables a user to make technically and administratively correct decisions for a stated purpose". In addition to the statistical data produced by testing specific parameters of the validation process, cost and applicability should be considered as described by IUPAC.
This very versatile concept of method validation enables the analysts to adapt the analytical methodologies to be suitable for the different applications. For instance, there is not any need to calculate the method detection limit (MDL) for a method used to determine the concentration of certain compounds when these compounds are expected at high concentrations (Boqué et al., 2002). In contrast, calculation of the method quantitation limit (MQL) is relevant issue in the case of residue analysis. Consequently, there is not any single package of specific requirements of the validation process suitable for every analytical application.
Basically in the validation process, complete analytical procedure including instrumental signal, representative sampling, sample preparation and clean-up procedure must be included. Any other parameters which have a relevant effect on the method performance should be taken into account. Whole concentration ranges in which the analytes may be present in the test samples must be covered. Based on the above introduction; recoveries, accuracy, precision (repeatability and intermediate precision), method detection and quantitation limits (MDL, MQL), linearity and matrix effects was achieved during the method validation as the most important parameters (Peters et al., 2007).

3.8.2 Operational conditions

3.8.2.1 Fortification experiments

Recoveries, method detection and quantitation limits were determined in surface water samples spiked with 5 and 25 µg/L of the target compounds using HPLC/UVD. The water samples were extracted by solid phase extraction using SDB 1 cartridges at pH 2.3.

Using LC/MS/MS; recoveries, accuracy, precision (repeatability and intermediate precision), method detection and quantitation limits as well as matrix effects were also determined at lower concentration levels. Surface water samples were spiked with the target compounds at 0.1, 0.2 and 1 µg/L, extracted using SDB1 at pH 2.3. The average recovery, precision and accuracy for each of the target compounds were calculated.

These parameters were determined for each analytes in liquid pig manure samples. For repeatability and intermediate precisions, a series of samples were fortified with analytes at different spiking levels. The target compounds were spiked at 2, 20 and 200 µg/kg in liquid pig manures samples, extracted by ethyl acetate at pH 9.5. The raw extracts were cleaned up by SEC only as single clean-up step in the preliminary work. The average recovery, precision and accuracy for each of the target compounds were calculated in one day to check repeatability and 3 consecutive days for intermediate precision. All the mentioned parameters were determined again for each analyte extracted by direct solvent extraction using ethyl acetate at pH 9.5 using two clean-up steps (SEC and SPE). For this purpose, four spiking levels including 2, 10, 50 and 100 µg/kg were used. For reduction of the workload, intermediate precision (combination of within and between days) was calculated as average of calculated relative standard deviations (RSD) of different spiking levels. RSD % of 4 replicates for one concentration level were determined in one day, combined with the means of RSD% of other spiking concentrations levels determined in another 2 consecutive days. The spiking levels of 10, 40 and 100 µg/kg were used to determine the recoveries, accuracy, and precision, of the target compounds extracted after lyophilization using methanol as extractant at pH 6.8. Recoveries, accuracy and precision (repeatability and combined intermediate precision) studies were also carried out using manure samples extracted after lyophilization by USE using methanol/ethyl acetate mixture (1:4) at pH 9.5. The spiking levels were 4, 10 and 50 µg/kg fresh manure.

In soil samples extracted by direct solvent extraction using methanol/ethyl acetate mixture (1:4) at pH 9.5, recovery, accuracy, precision (intra-day, repeatability), method detection and quantitation limits, linearity and matrix effects were determined. Different spiking levels were used to achieve these parameters as following: 2, 4, 40 and 100 in sand soil and 4, 40 and 100 µg/kg in clay soil. In manured soil samples extracted by direct solvent extraction using methanol/ethyl acetate mixture (1:4) at alkaline pH, the target compounds were spiked at 2, 10, 50 and 100 µg/kg in manured sand soil and at 2 and 100 µg/kg in manured clay soil. In manured soil samples extracted using methanol/ethyl acetate mixture (1:4) at alkaline pH and USE, the target compounds were spiked at 2, 10 and 100 in manured sand soil and at 2 and 50 µg/kg in manured clay soil.

Recoveries (R) were determined by comparing the concentrations of the samples spiked before extraction with the samples spiked after extraction using Eq. 3.1 and external calibra-

tion curves (Yang et al., 2004; Kim and Carlson, 2007). These calibration curves were recorded from 5 pg to 5000 pg/µL with correlation coefficient ≥ 0.995. Considering the losses during the clean-up procedure, recovery were calculated and presented as absolute or relative recovery. Based on the Eq. 3.1, the obtained recovery when the concentration of the samples (zero samples) spiked before extraction (S1-S4) compared with the concentration of the sample spiked after extraction but before the clean-up procedure (B1 and B2) is defined as relative recovery (extraction efficiency). The obtained recovery when the calculated concentration of sample spiked before extraction (S1-S4) compared with the calculated concentration of the sample spiked after clean-up procedure (B3 and B4) is defined as absolute recovery. The losses during clean-up procedure can be also calculated via comparing the mean concentration of B1 and B2 with the mean concentration of B3 and B4. Moreover, matrix effects (ME %) could be identified at every spiking levels when peak area of B3 and B4 compared those of standard solution containing analytes of identical concentrations. This experimental design was also used in all the other extraction procedures and different matrices.

$$\text{Recovery} = \frac{\text{Concentration of the samples spiked before extraction}}{\text{Concentration of the samples spiked after extraction}} .100 \qquad \text{(Eq.3.1)}$$

In each case, samples were analyzed at least in triplicate and blanks were analyzed in order to test background contamination and to check any interference for these analytes to ensure the chromatographic selectivity of this method.

3.8.2.2 Accuracy and precision

Accuracy and precision were checked for specified concentration ranges using the optimized analytical procedures for analysis of the target compounds in different matrices as mentioned in section 3.8.2.1. Accuracy could be presented as percent recovery or as the difference between the mean of measured values and the true value which is known as relative errors. In terms of bias or relative errors, accuracy was calculated for each analyte (RE) as the percent deviation of the mean of all calculated concentration values at a specific level from the spiked concentration as it can be seen in Eq. 3.2 (Maraschiello et al., 2001; Nobilis et al., 2007; Peters et al., 2007) The concentrations of the analytes in fortified samples were calculated via external calibration curves and Eq. 3.1.

$$RE\ (\%) = \frac{(\text{Mean calculated concentration} - \text{true concentration})}{\text{true concentration}} . 100 \qquad \text{(Eq. 3.2)}$$

The accurate method must be precise otherwise accuracy will lose its meaning in the validation process. Thus, precision is important for trusting in the output of the validation process. Precision is expressed as relative standard deviations (RSD %). To confirm the acceptance of the observed precision 3-4 replicates at a minimum of 3 spiking levels were tested as recommended by the International Conference on Harmonization Q2B (ICH, 1996) and European Commission Decision 2002/657/EG (EC, 2002) guidelines. The values of accuracy, repeatability and intermediate precision were accepted when RSD was ≤ 20 % and recovery was in the range ± 20 % of the nominal values (Shah et al.,1992, 2000).

3.8.2.3 Method detection limits and method quantitation limits

To calculate instrumental detection limits (IDLs) and instrumental quantitation limits (IQLs), the target compounds were injected into LC/MS/MS system at different concentrations started from 0.2 pg/µL up to 10 pg/µL. IDL and IQL are defined as the absolute analyte concentrations of standard solutions which give a signal to noise ratio of 3 and 10, respectively. The signal to noise ratio was calculated by analyst software version 1.5. However, method detection (MDLs) and quantitation limits (MQLs) were matrix based and determined from spiked samples.

The spiked concentration used to calculate the method detection limit is the minimum reproducible concentration of a substance that can be measured according to the US Environmental Protection Agency (EPA, 1984). A 70 % to 110 % recovery range with RSD ≤ 20 % is the parameter to fulfill the method requirements as explained by Wisconsin Department of Natural Resources (1996). Here, MDL and MQL were determined by analyses of target compounds spiked into different matrices, i.e. surface water, liquid pig manure, soils and manured soils. Surface water sample were spiked with target compounds at 5 and 0.1 µg/L using HPLC/UVD and LC/MS/MS respectively. The target compounds were spiked at 2 µg/kg in the other matrices. Principally, 7 samples were extracted, cleaned-up and finally analyzed as described before. Based on the standard deviation (SD), the MDL was calculated according to the equation 3.3:

$$MDL = t\ (n-1,\ 1-\alpha = 0.99) . (SD) \qquad \text{(Eq. 3.3)}$$

Where,
t (n-1, 1-α = 0.99): students t value appropriate for a 99 % confidence level and the estimation of the standard deviation with n-1 degrees of freedom (t = 3.143, n = 7).
SD: standard deviation of the replicate analysis (n = 7), while MQL = SD x 10.

The following inequalities are useful for evaluating the calculated MDL:
MDL < spike Level < 10 times of the calculated MDL.

3.8.2.4 Matrix effects

The matrix effects are practical challenges for LC/MS/MS directly dependent on the selection of calibration method used for quantitation purposes. Matrix effects (ME) are changes of analyte responses which may appear in the form of signal enhancement or signal suppression. As consequences of these unpredicted effects, the accuracy and precision of the analytical method can be affected. Several methods have been described in the literatures to evaluate the matrix effects in different matrices. Two methods were used in this study to identify and calculate the matrix effects. First was applied in surface water as proposed by Matuszewski et al. (2003) Van de Steene et al., (2006) and Gosetti et al., (2010). Three series of samples were prepared at low and high spiking concentration levels. Samples in series A consisted of benzimidazole standard solutions prepared in methanol at concentrations of 0.1 and 1 µg/L for all of the target compounds except FEN and FLU-M6. Those concentrations were 0.05 and 0.5 µg/L and 0.2 and 2 µg/L for FEN and FLU-M6, respectively. For preparation of the samples in series B, blank surface water samples were first extracted as described before. Then, the evaporated residues were reconstituted in 1 mL methanol containing the analytes at same concentrations as in series A. In series C, blank surface water samples were spiked with the target compounds at the same concentrations as in series A and B. Thereafter, they were extracted and the evaporated residues were reconstituted in 1 mL methanol. When analyte peak areas of the LC/MS/MS analyzed samples in series A were compared with those measured in series B, the ratio ((B/A) × 100) is defined as the absolute matrix effect (ME%). A value of 100 % indicates that there is not any absolute matrix effect. There is a signal enhancement if the value is > 100 % and signal suppression if the value is < 100 %. The recoveries were calculated by comparing the peak areas from series C with those from series B, where the ratio ((C/B) × 100) is defined as the absolute recovery (R). In this case the calculated recoveries are not influenced by matrix effect. Finally, process efficiencies (PE) were estimated by comparing of the peak areas from series C to those from series A and also reported in %. These effects were calculated by comparing between the analyte

responses in the solvent and in the matrix under study using identical instrumental conditions and the next equations:

$$ME\ (\%) = \frac{B}{A} \cdot 100 \quad \text{(Eq. 3.4)} \qquad R(\%) = \frac{C}{B} \cdot 100 \quad \text{(Eq. 3.5)} \qquad PE = \frac{C}{A} \cdot 100 \quad \text{(Eq. 3.6)}$$

Second method was applied on the other matrices. The slopes of the calibration curves which were prepared in the solvent using known serial dilution of analyte standard solutions were compared with those prepared in the matrices under study (Gros et al., 2006). Matrix-matched calibration curves were prepared in matrix standard solutions. For this purpose, the zero manure samples were extracted using ethyl acetate at pH 9.5 and cleaned-up using SEC and SPE. The obtained calibration curves were compared with those achieved for the same standards at same concentrations in methanol (external calibration). The chloro-analogue of FLU was used as internal standard and added during preparation of these calibration curves to evaluate efficiency of internal calibration method to correct these effects in the same experiments (internal calibration). For matrix-matched and external calibrations, the analytes concentrations were plotted versus the corresponding peak areas of the analytes or areas ratios in case of internal standard calibrations. The compounds were not subjected to matrix effects when both curves prepared in manure matrix and external, i.e., solvent based calibrations are parallel and totally overlapped. Larger or smaller slopes of matrix-matched calibration curves than solvent based calibration curves indicate that these compounds are subjected to signal enhancement or signal suppression, respectively. For external calibration curves, calibration points at 100, 200, 300, 500 and 700 pg/µL were made by serial dilution of the stock standard solutions in methanol. In the matrix-matched calibration, the calibration standard solutions (1 mL) were filled into 2-mL volumetric flasks and 1-mL manure extracts were added. This work was repeated to study the matrix effects in manure samples extracted by methanol after lyophilization as well as soil and manured soil samples extracted by direct solvent extraction method using the methanol/ethyl acetate mixture (1:4 v/v) at alkaline pH. The internal standard was added to the calibration standards at 50 pg/µL in manure extract, while, in soil and manured soil extracts, its concentration was 40 pg/µL. The manure, soil and manured soil matrices were prepared by the established extraction methods described before. The calibration curves were calculated using a weighted (1/X) linear regression model.

3.8.2.5 Quantitation methods

External calibration

Quantitation of the target compounds during fortification experiments was based on external calibration curves of benzimidazole compounds recorded in methanol and ranged from 2.5 to 1000 and 5 to 5000 pg/µL combined with Eq.3.1 in case of water and the other analyzed matrices, respectively. LC/MS/MS was operated in the positive ion mode (PI) using ESI.

Multiple standard additions

Known volume of liquid pig manure samples containing 1 to 2.5 g ds were weighed. These samples were extracted by direct solvent extraction using ethyl acetate and after lyophilization using methanol. The raw extracts were cleaned-up as described before. Equal volumes of the clean extract solutions were taken and separately spiked with different amounts of the analytes standard solutions at 100, 200, 300, 500, 1000 pg/µL. Thus, 5 calibration solutions were prepared containing the extracts of manure samples. All spiked sub-samples were diluted to the same volume to finally record multiple standard addition calibration curves following the method described by Nerín et al. (1994).

The instrumental responses were then determined for all these solutions and the results plotted versus the added concentrations. The unweighted regression line was calculated in the normal way and extrapolated to the point at which y = 0. The point at zero added concentration of the target compounds is the concentration of the unknown samples, and the other points are the added concentrations plus the background concentration. The absolute value of the x-intercept is the concentration of the analytes in unknown samples. The standard addition concentration was chosen based on the estimated concentration via external calibration in one of the real samples at the beginning of real samples analysis.

Single point standard addition

The second method was single point standard addition. Six sub-samples, each one has the same volume of liquid pig manure samples, were prepared and extracted by LLE using ethyl acetate at pH 9.5. Two sub-samples were spiked after extraction immediately before clean-up procedure with known concentration of the target compounds where the concentrations of non spiked samples were calculated according to equation 3.7 or 3.8 (Ito and Tsukada, 2002; Zenkevich and Klimova, 2006; Garrido Frenich et al., 2009).

$$\frac{P_x}{P_{x+add}} = \frac{m_x}{(m_x + m_{add})} \qquad \text{(Eq. 3.7)}$$

$$m_x = \frac{m_{add} \cdot p_x}{p_{x+add} - p_x} \qquad \text{(Eq. 3.8)}$$

P_x and $P_{x\,+\,add}$ are chromatographic peak areas of the analytes before and after addition of known concentration, respectively, where m_x is the concentration of unknown sample m_{x+add} is the concentration of the added concentration plus sample concentration. This procedure was also used to calculate the concentrations of the detected compounds in two selected real samples extracted via USE method, where the obtained results were compared with those obtained using external and internal calibrations.

3.9 Extraction efficiency and aging tests

Pig manure produced in intensive farming activity are usually stored in a manure tanks in the range of 90-180 days prior to spreading on agricultural land (Schlüsener et al., 2006). Fenbendazole and flubendazole with corresponding metabolites may be dispersed with manure produced by treated animals unless degradation (biotic or a biotic) takes place during manure storage. The extractability of these benzimidazoles after liquid manure storage under different field conditions could be affected by the time mainly due to sorption-desorption equilibrium and microbial effects (Löffler and Ternes, 2003). This test was designed to study aging effects on the extraction efficiency of FEN, FLU and corresponding metabolites from pig manure after 30 days incubation period by direct solvent extraction. This experiment was carried out under anaerobic conditions at 20 ºC and 4 ºC as well.

Six series of liquid pig manure samples containing 5 % (ds), each one consisted of blank and 4 samples (50 g) were filled in 300-mL Erlenmeyer flasks. An aliquot of the stock standard solution containing FEN, FLU as well as corresponding metabolites, was added to each 300-mL Erlenmeyer flask resulting in a final concentration of 100 µg/kg fresh manure. The concentration of FEN and FLU-M6 were half and double of these spiked concentration, respectively. The flasks were left open for 1 h to evaporate the solvent and to avoid toxic effects on microorganisms of the manure samples.

All flasks were purged with nitrogen to guarantee anaerobic conditions and incubated in the dark. 3 series were incubated at 4 ºC and 3 series at 20 ºC in order to gather information on the temperature depending effect of biotransformation. At each sampling time (0, 15 and 30 days), 5 samples were prepared for analysis. Samples were buffered by ammonium chloride buffer pH 9.5 and extracted by mechanical shaking with ethyl acetate as described before. The raw extracts were cleaned by SEC. 7 µL were injected into LC/MS/MS.

3.10 Analysis of real pig manure samples

In order to test the applicability of the developed methods for analysis of the target compounds in real samples, 7 pig manure samples (PM1-7) were collected via grab sampling from manure cellars. These samples were taken at seven pig fattening farms in the catchment area of the Chamber of Agriculture, Oldenburg, Germany. FLU was applied as a food additive at 5 mg/kg pig bw for 5 consecutive days as de-worming agent as described before.
Subsamples each one equal 22.5 and 27.5 g of fresh samples (equivalent to 2.5 g dry substance) of PM1 and PM5 respectively, were weighed into 300-mL Erlenmeyer flasks, and buffered with 25 mL ammonia/ammonium chloride (pH 9.5). Samples were extracted with 100 mL ethyl acetate on a horizontal shaker at 220 rpm for overnight. The samples were rinsed two times with 100 mL and 50 mL for 60 and 30 min, respectively.
The combined raw extracts were rotary evaporated, then micro-filtered and finally cleaned-up as mentioned before. The concentrations were calculated using multiple standard addition technique. The entire extracts of each sample eluted in one round bottom flasks, evaporated under a gentle nitrogen stream and then reconstituted in 5 mL methanol. Later on, these extracts of the real matrix, were used to record the multiple standard addition calibration curves using serial dilutions of the standard solution of the target compounds mixture, e.g., 100, 200, 300, 500, 1000 pg/µL.
This procedure was also applied for lyophilized PM1 and PM5 samples and methanol as extractant without pH adjustment. Moreover, the concentrations of these two samples were recalculated with the other five samples (PM1-PM7) using single point standard addition technique and ethyl acetate as extractant at pH 9.5. During this, replicates of PM1 to PM7, each one contains a known volume of well homogenized fresh sample equivalents to about 0.5 to 1g dry substance from each sample were extracted, cleaned-up and finally LC-MS-MS analyzed. Ultrasound-assisted solvent extraction (USE) using methanol/ethyl acetate mixture (1:4) was also used to extract the target compounds in lyophilized PM1 and PM5 samples at pH 9.5. The concentration was calculated using single point standard addition technique, external and internal calibration.

3.11 Identification and confirmation techniques

The use of the triple stage quadrupole instrument (QqQ) using MRM of 4000 QTRAP provides a method with high sensitivity and selectivity. For identification and quantitation purposes, 2 transitions were selected to build MRM method for each compound. According to European Commission Decision 2002/657/EG (EC,2002) for confirmation and identification

of organic pollutant in live animals and animal product, 3 identification points (IP) are required for legally used compounds and 4 identification points for illegal compounds must be achieved. Number of identification points depends on the technique by which the analyte of interest was analyzed (**Table 3.8**).

Table 3.8: The relationship between analysis techniques and numbers of identification points according to Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (EC, 2002).

Mass spectrometer	Identification point per ion
Low resolution mass spectrometry (LR) - LR-MS^n Precursor ion - LR-MS^n Transition products	 1 1.5
High resolution mass spectrometry (HR) - HR- MS^n Precursor ion - HR-MS Transition products	 2 2.5

To get highly qualified identification points, other requirements such as selection of specific ions (fingerprint ions) and chromatographic behavior (retention time) should be achieved. The ion ratios of selected transitions using reference standard with those obtained using spiked or unspiked real samples should be compared, where the maximum permitted tolerance depends on the relative intensities of selected ions (**Table 3.9**).

Table 3.9: Maximum permitted tolerances for relative ions intensities using different spectrometric techniques according to Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (EC, 2002).

Relative intensity (of base peak)	GC/MS-EI	GC/MS-CI, $GC/MS/MS^n$ LC/MS, $LC/MS/MS^n$
$>$ 50%	± 10 %	± 20 %
20 to 50 %	± 15 %	± 25 %
10 to 20%	± 20 %	± 30 %
$\leq$ 10%	± 50 %	± 50 %

In addition to the above regulations, mass spectra produced by IDA method using MRM as full scan and EPI as dependent scan were used to support the confirmation of the positive results during analysis of real samples.

4. Results and discussion

The objectives of these research activities focused on the development of a novel method for the simultaneous analysis of FEN and FLU as well as their corresponding metabolites in liquid pig manure and manured soil. For this purpose preliminary tests were conducted for surface water and soil to study the analytes properties during experimental handling with reduced matrix complexity. Thus, different extraction and clean-up procedures were checked and subsequently transferred to manure and manured soil matrices. Besides different sample preparation techniques, different detection techniques for identification and quantitation of the target compounds under study were applied. Due to the intermediate to high polarity of the analytes, they based on HPLC/UVD or HPLC/DAD and LC/MS/MS. For the latter, ESI and APCI techniques were applied. Consequently, versatile measures of analytical quality assurance were performed focused on different quantitation techniques and compensation of matrix effects. Besides fortification experiments, reference-manure samples from anaerobic biotransformation test under laboratory conditions were investigated in order to take the aging of benzimidazole residues into account. Finally, real manure samples from different pig fattening farms were analyzed and results were interpreted utilizing already published data for some of the analytes in different sample matrices.

4.1 Extraction procedure

The analysis of organic pollutants in environmental samples is a complicated procedure. The target compounds under study occur either at low ng/L-concentrations as, e.g., in water samples or at µg/kg-concentrations but in complex sample matrices, e.g., liquid manures or manured soils. This is why sample preparation, including sample extraction and clean-up procedures, is one of the most relevant steps. In order to determine trace amounts of pharmaceutical compounds present in samples under analysis, the analytes are to be exhaustively extracted out of the sample matrixes without simultaneously releasing too many co-extractants. Several extraction techniques, therefore, were applied and stepwise advanced for this purpose. Based on physicochemical properties of the analytes and sample matrices, one of several methods can be selected. For liquid samples, solid phase and liquid–liquid extraction (LLE) extraction are frequently used. Pressurized liquid extraction (PLE), Soxhlet, microwave-assisted extraction (MAE), supercritical fluid extraction (SFE) and ultrasound-assisted solvent extraction (USE) are widely used for the extraction of solid samples. Among these extraction techniques solid phase extraction, direct solvent extraction as well as ultrasound-assisted extraction were successfully used in this study for water, liquid pig manure, soil and manured soil samples.

4.1.1 Surface water samples

Simultaneous extraction of the target compounds which have various physico-chemical properties such as compounds from different classes or compounds with their metabolites is the main analytical challenge in any analytical protocol. The selection of the suitable sorbent for solid phase extraction to reach high extraction efficiencies for these compounds is the first step. Therefore, styrene-divinylbenzene (SDB1) and Oasis HLB cartridges (hydrophilic-lipophilic balance) as examples of polymeric sorbents were checked for extracting the target compounds from surface water samples. Results of these experiments are summarized in **Figure 4.1** to **4.3.**

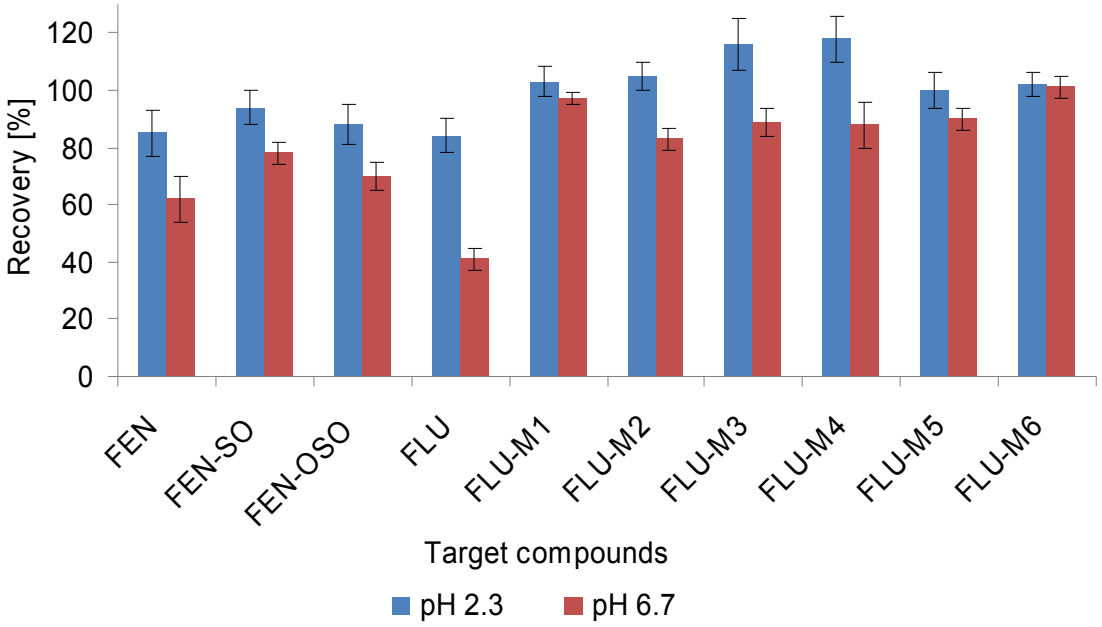


Figure 4.1: Recovery rates of fenbendazole and flubendazole with corresponding metabolites spiked at 1 µg/L to 200-mL water samples of the Oker River using styrene-divinylbenzene (SDB1) cartridges at two pH values. Relative standard deviations (RSD) are given as error bars (n=4).

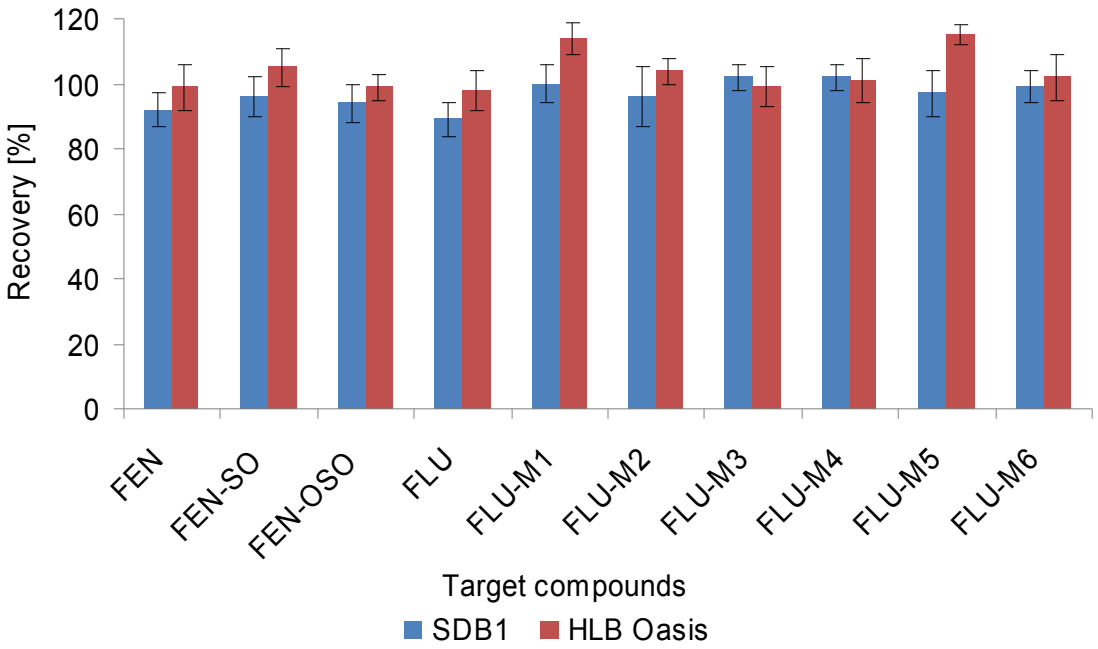


Figure 4.2: Recovery rates of fenbendazole and flubendazole with corresponding metabolites spiked at 1 µg/L to 200-mL water samples of the Oker River at pH 2.3 using different sorbents. Relative standard deviations (RSD) are given as error bars (n=4).

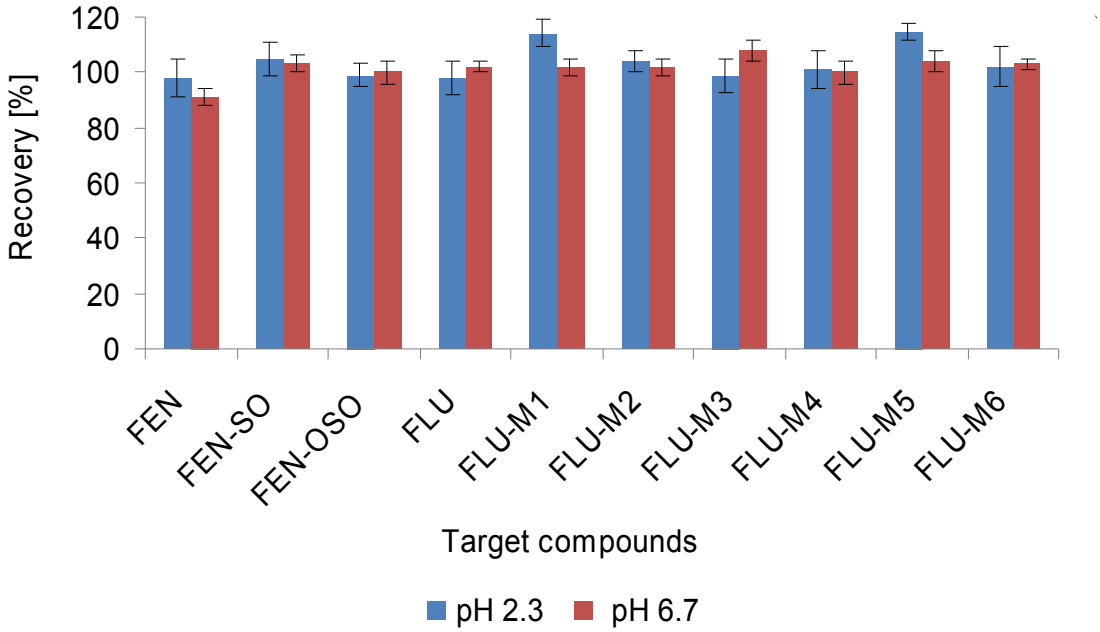


Figure 4.3: Recovery rates of fenbendazole and flubendazole with corresponding metabolites spiked at 1 µg/L to 200-mL water samples of the Oker River using HLB Oasis cartridges at two pH values. Relative standard deviations (RSD) are given as error bars (n=4).

High recovery rates for the target analytes ranged from 91 to 115 % obtained using Oasis HLB cartridges at acidic and neutral pH. This attributed to Oasis HLB cartridges consisting of sorbent with an equal ratio of the hydrophilic N-vinylpyrrolidone and the lipophilic divinylbenzene with wide range of stability from pH 1-14. Those are able to extract the compounds under investigation even though they have different physicochemical properties (Hao et al., 2006). These target compounds can interact with either the hydrophilic or the lipophilic moieties of the HLB sorbent. These characteristics can eliminate the need for different pH adjustment of the samples to achieve efficient extraction allowing quantitative determination of these compounds at ng/L-concentrations. Adequate extraction efficiencies were obtained using styrene-divinylbenzene cartridges when water samples were adjusted at acidic pH values. Thus, recovery rates using SDB1 cartridges were 84 to 118 % for all the target compounds at pH 2.3. According to the European Commission Decision 2002/657/EG (EC, 2002), these recovery rates were within the acceptable recovery range of 70 to 120 %. However, these results were in opposite to results for piperazinylic quinolones obtained by Turiel et al. (2003) extraction. In his study, those basic compounds can extracted with high recovery rates only at basic conditions. The possible explanation for this efficient extraction of the target compounds under study at pH 2.3 is the existence of non-specified modifications of the polystyrene-divinylbenzene matrix, e.g., light sulfonation (Weigel et al., 2004). Based on the reported data, benzimidazole compounds are zwitterions (Kim et al., 2010) or weakly basic molecules (Danaher, 2003). Under suitable conditions, these molecules may be protonated at acidic pH, where this property, in order to get efficient extraction, is essential for retaining these basic compounds by this (modified) cationic exchange resin. When the samples extracted at the original pH (6.7), high extraction efficiency was still provided by SDB1 cartridges. The recoveries ranged from 70 to 101 % for all the target compounds except the parent compounds FEN and FLU, where their recoveries were 62 and 41 %, respectively. This may be attributed to polystyrene-divinylbenzene resin copolymer as hydrophobic resin has better analyte retention, mainly for highly polar compounds, due to relatively large number of active aromatic sites which allow π-π interaction (Turiel et al., 2003; Żwir-Ferenc, 2006). The obtained results using SDB1 cartridges at neutral pH are consistent with the results obtained by Balizs (1999). In that study, FEN, FEN-SO, FEN-OSO, FLU with 11 other benzimidazole compounds were extracted from pig tissue samples using ethyl acetate at alkaline pH. These extracts were evaporated, acidified using 0.2 M HCl and then finally washed using n-hexane. Thereafter, acidic layers were evaporated, exchanged by an ammonium acetate/methanol mixture (1:1), loaded on SDB1 cartridges as the main clean-up step in this procedure. Finally, the elution was carried out with 3 mL methanol/ethyl acetate (1:4). The recoveries were 44, 75, 54 and 50 % for FEN, FEN-SO, FEN-OSO and FLU, respectively. The recovery rates for the other

compounds ranged from 8 to 69 % except two compounds recovered at 81 and 117 %. These results indicated that the pH value plays an important role for enhancement of the retention of these compounds using these cartridges.

Desorption of the target compound is also critical step in solid phase extraction method. Therefore, pH values of the eluents were also tested. Slightly or no differences in the recovery rates were observed when methanol acidified with 1% of formic or pure methanol were used for the elution of the target compounds. 5 mL methanol were appropriate for complete elution of these analytes especially when 200-mg SDB1 cartridges were used (Weigel et al., 2004). Considering the variability of environmental matrices, therefore, as margin of safety 2 x 5 mL pure methanol was used in this work. After the identification of acidic pH as the best condition for the analytes extraction the analytical method development for the extraction, separation and quantitation of these benzimidazoles from water samples mainly focused on the use of SDB 1 cartridges followed by HPLC-UVD and LC/MS/MS analysis. **Figure 4.4** shows the analytical scheme of the optimized method for analysis of target compounds in surface water samples. Subsequently, this SPE technique was subsequently tested for clean-up of manure, soil and manured soil samples.

4.1.2 Manure samples

Extraction procedures for benzimidazoles from liquid manure or manured soil are only applied within 2 studies until today. First one was performed by Weiss et al. (2008) and referred to FLU and its metabolites FLU-M1 and FLU-M2. In this procedure manure samples (containing 1.1–2 % dry substance) were diluted 1:1 with a methanol/water mixture (1:1, 1 % acetic acid). After centrifugation at 2000 rpm for 20 min, these extracts were directly analyzed using LC/MS/MS. Kreuzig et al. (2007) extracted FLU and FEN from liquid pig manure based on the direct solvent extraction with ethyl acetate at pH 5.2. Because none of both extraction procedures did not involve the 10 target benzimidazoles under study different extraction procedures were additionally checked.

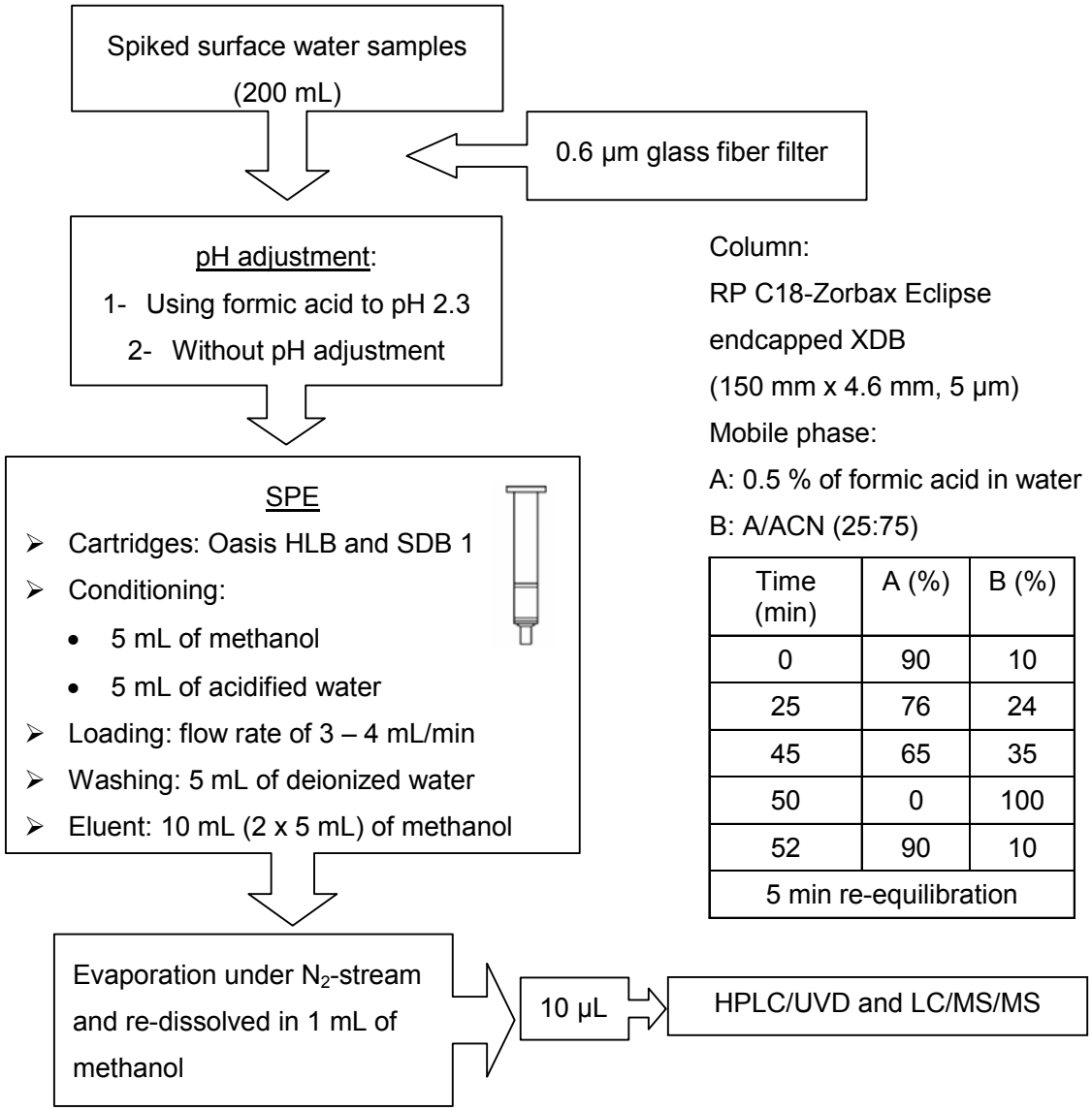


Figure 4.4: Analytical scheme of the optimized method for analysis of benzimidazole compounds in surface water using Oasis HLB and styrene-divinylbenzene (SDB1).

4.1.2.1 Direct solvent extraction

Based on direct solvent extraction of target benzimidazoles using ethyl acetate, series of extraction experiments at different pH values were conducted in order to achieve an exhaustive extraction procedure. For this purpose, several parameters were needed to be optimized, e.g., number of extraction cycles, extraction time, pH adjustment and salting out effect. The initial conditions for the direct solvent extraction were modified from the procedure adopted by Kreuzig et al. (2007) to extract the parent compounds from liquid pig manures.

Four replicates, each one was 50 g liquid manure, were spiked with the target compounds at 100 µg/kg fresh samples. These were extracted in 3 consecutive steps. These include shaking overnight with 100 mL ethyl acetate at 220 rpm with two additional rinsing steps 100 mL and 50 mL with 60 min and 30 min shaking times, respectively. Raw extracts were combined, rotary evaporated, cleaned up by SEC and finally analyzed using LC/MS/MS.

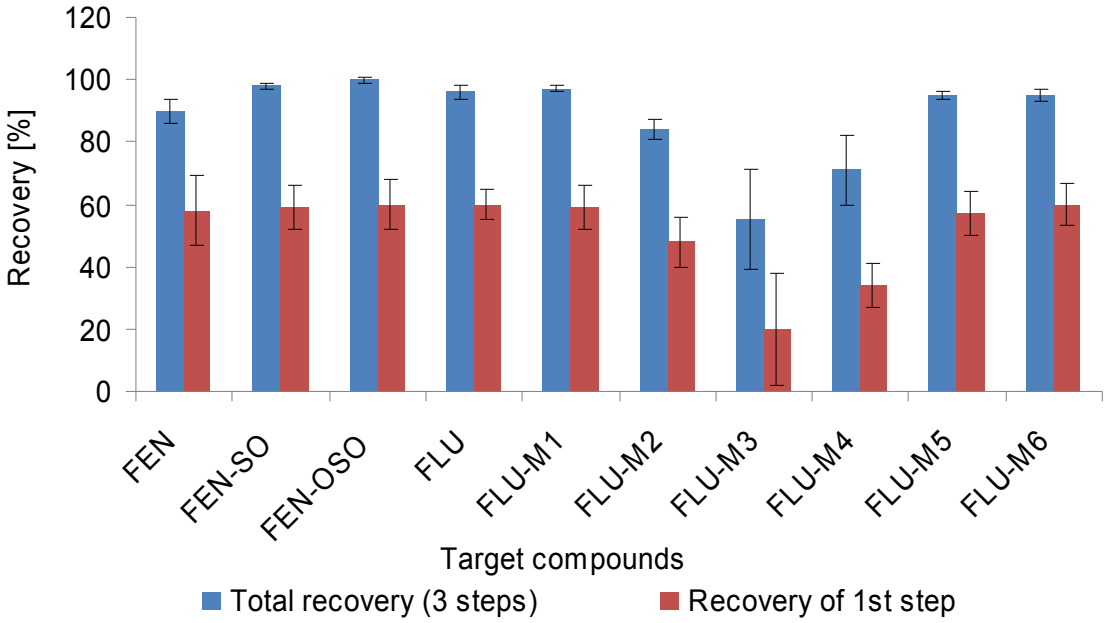


Figure 4.5: Recovery rates of fenbendazole and flubendazole with corresponding metabolites obtained by first extraction step vs. one extraction step combined with 2 rinsing steps (total) in liquid manure at original pH 6.8.

It was found that recovery rates of 70 to 120 %, defined as acceptable recovery rates by the European Commission Decision 2002/657/EG (EC, 2002), can be reached by the direct solvent extraction shaking the manure sample with 100 mL ethyl acetate overnight. However, the extraction step had to be followed by 2 rinsing steps in order to transfer the extracted analytes from the extraction medium into fresh solvent. As shown in **Figure 4.5**, all target compounds were extracted at recovery rates ranging from 71 to 100 %. However, FLU-M3 and FLU-M4 were not quantitatively extracted due to recoveries of 55 and 70 %, respectively. In order to improve the extraction of these compounds, several parameters were optimized. First parameter optimized was pH which is considered as the relevant parameter to maximize or minimize the efficiency of organic solvent extraction of environmental samples. Different pH including neutral (6.8 ± 0.1), acidic (4.4 ± 0.1) and alkaline pH (9.5 ± 0.1) were tested. It was found that the efficiency of the extraction method for all target compounds increased with the increase of the pH up to 9.5.

These results may be attributed to the benzimidazole compounds have 2 pK_a values, e.g., at

pH < 6.0, these compounds may be positively charged and at pH > 10 they may be negatively charged (Danaher et al., 2007; Msagati et al., 2006). In between these compounds are neutral and more hydrophobic. This neutral region corresponded to the optimum pH at which efficient extraction can be achieved. Therefore, pH 9.5 was the best pH value to get a method with highest extraction efficiency. Recovery rates were higher than 70 % for all investigated compounds. **Table 4.1** shows the recoveries of the target compounds at different pH.

Table 4.1: Recoveries of fenbendazole and flubendazole with corresponding metabolites of spiked liquid manure samples 100 µg/kg at different pH calculated using matrix-matched calibration curves.

Analytes	pH 6.8	pH 4.4	pH 9.5
FEN	87 ± 6	88 ± 3	79 ± 5
FEN-SO	86 ± 6	87 ± 5	80 ± 5
FEN-OSO	86 ± 7	86 ± 3	80 ± 3
FLU	85 ± 7	86 ± 3	80 ± 3
FLU-M1	85 ± 11	85 ± 4	79 ± 6
FLU-M2	73 ± 14	69 ± 5	70 ± 6
FLU-M3	50 ± 21	25 ± 10	70 ± 5
FLU-M4	70 ± 12	54 ± 4	79 ± 13
FLU-M5	76 ± 7	85 ± 4	78 ± 9
FLU-M6	83 ± 7	83 ± 4	77 ± 5

As it can be seen**,** all the target compounds were quantitatively extracted at alkaline pH, but FLU-M2 and FLU-M3 were still at lowest acceptable recovery range. Considering the losses during the cleanup procedure which cannot be compensated using matrix-matched calibration curves, the recoveries were calculated using solvent calibration curves and Eq. 3. The improvement in the recovery rates ranged from 4 to 17%. This amount is equivalent to the lost amount during the cleanup procedure as shown in **Figure 4.6**.

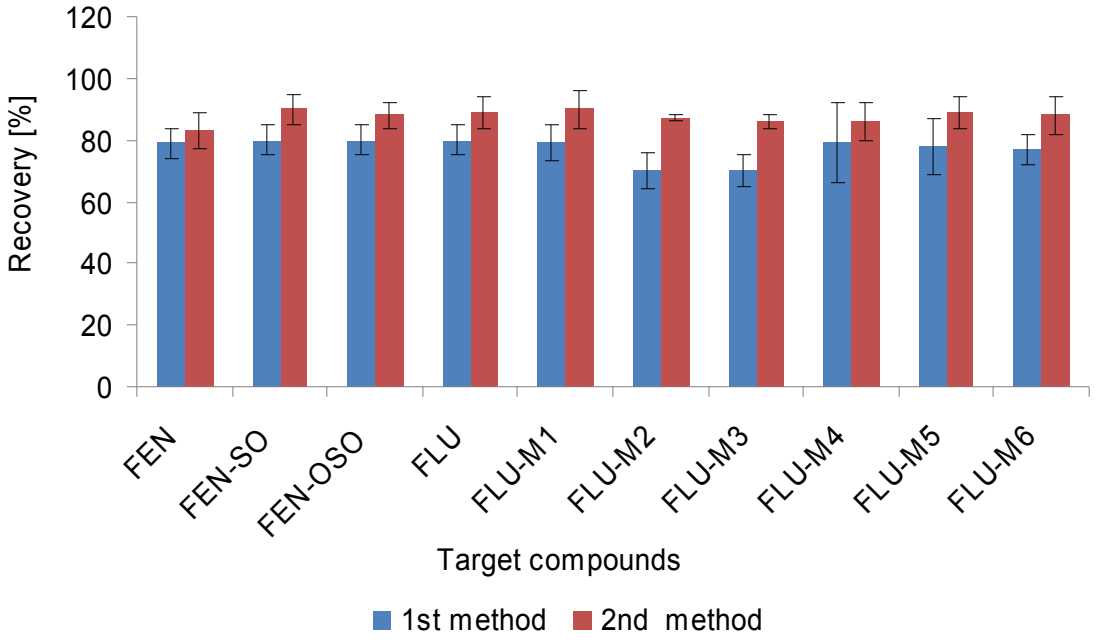


Figure 4.6: Recovery rates of fenbendazole and flubendazole with corresponding metabolites calculated using matrix-matched calibration curves (1st method) and external calibration curves with Eq. 3.1 (2nd method).

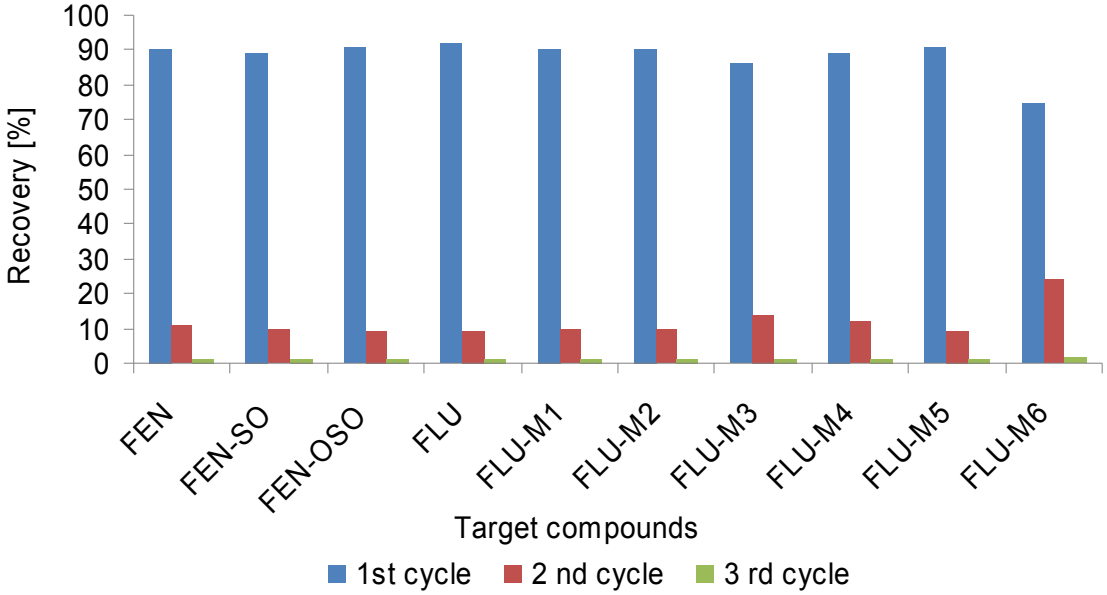


Figure 4.7: Recovery rates of fenbendazole and flubendazole with corresponding metabolites spiked in liquid manure samples.

After identification of pH as relevant parameter to achieve exhaustive extraction procedure, it was necessary to restudy the actual number of extraction cycles required to make this procedure exhaustive. It was found that the first extraction cycle was sufficient to realize high

recovery rates for all compounds. The obtained recoveries were in the range from 86 to 90% for all target compounds except for FLU-M6 it was 75 %. Observed increase of FLU-M6 recovery rate from 75 to 99 % with slightly improvement for the others from 9 to 14% were obtained by the second one. For all compounds, recoveries were considered negligible in third cycle as it can be seen in **Figure 4.7**. For this reason, the last two cycles are considered as rinsing steps. However, these two steps are still required due to the variability of the manure matrices, where the magnitude of phase separation was different according to dry substance contents, detergent and other additives in the farms during manure sampling.

In fortification experiments, irrelevant differences were found between recoveries obtained after overnight (12 h) shaking with 100 mL ethyl acetate at pH 9.5 and recoveries obtained after 2, 4, 6 and 8 h shaken at the same conditions. However, in order to take into account aging process and higher matrix affinities of compounds, shaking overnight should be preferred. **Figure 4.8** shows the obtained recoveries of the target compounds at different shaking time during first extraction cycle at alkaline pH and ambient temperature.

Salting-out effect of added sodium chloride (10 g) to the samples prior extraction was additionally studied. However, there were no observed improvements in the obtained recoveries of the samples. For this reason this step was excluded from the final procedure.

The mean recoveries of the target compounds were calculated preliminary in manure samples at different spiking levels, e g., 4, 50, 100 and 500 µg/kg at original pH and 2, 20, 100 and 200 µg/kg at pH 9.5 as well as 100 µg/kg at acidic pH. The overall recovery rates were 55 to 101 %, 80 to 101 % and 25 to 88 % with RSD ≤ 18, 17 and 10 % respectively (**Appendix, Table A1**). During this work, it was found that extracts obtained using ethyl acetate at alkaline pH were cleaner than the extracts obtained at acidic pH. This is may be attributed to humic and fulvic acids are neutral and more hydrophobic at acidic pH. Thus, high amounts of humic substances could be extracted under this conditions using ethyl acetate. Therefore, it was necessary to add another clean-up step to make this extraction procedure suitable under different pH conditions. Later on, the suitability of the optimized procedure to efficiently extract the target compounds from liquid pig manure at different spiking levels was re-examined. Liquid pig manure samples were spiked with target compounds at 2, 10, 50 and 100 µg/kg. Samples were extracted with ethyl acetate at alkaline pH, cleaned up by SEC and SPE and finally analyzed by LC/MS/MS.

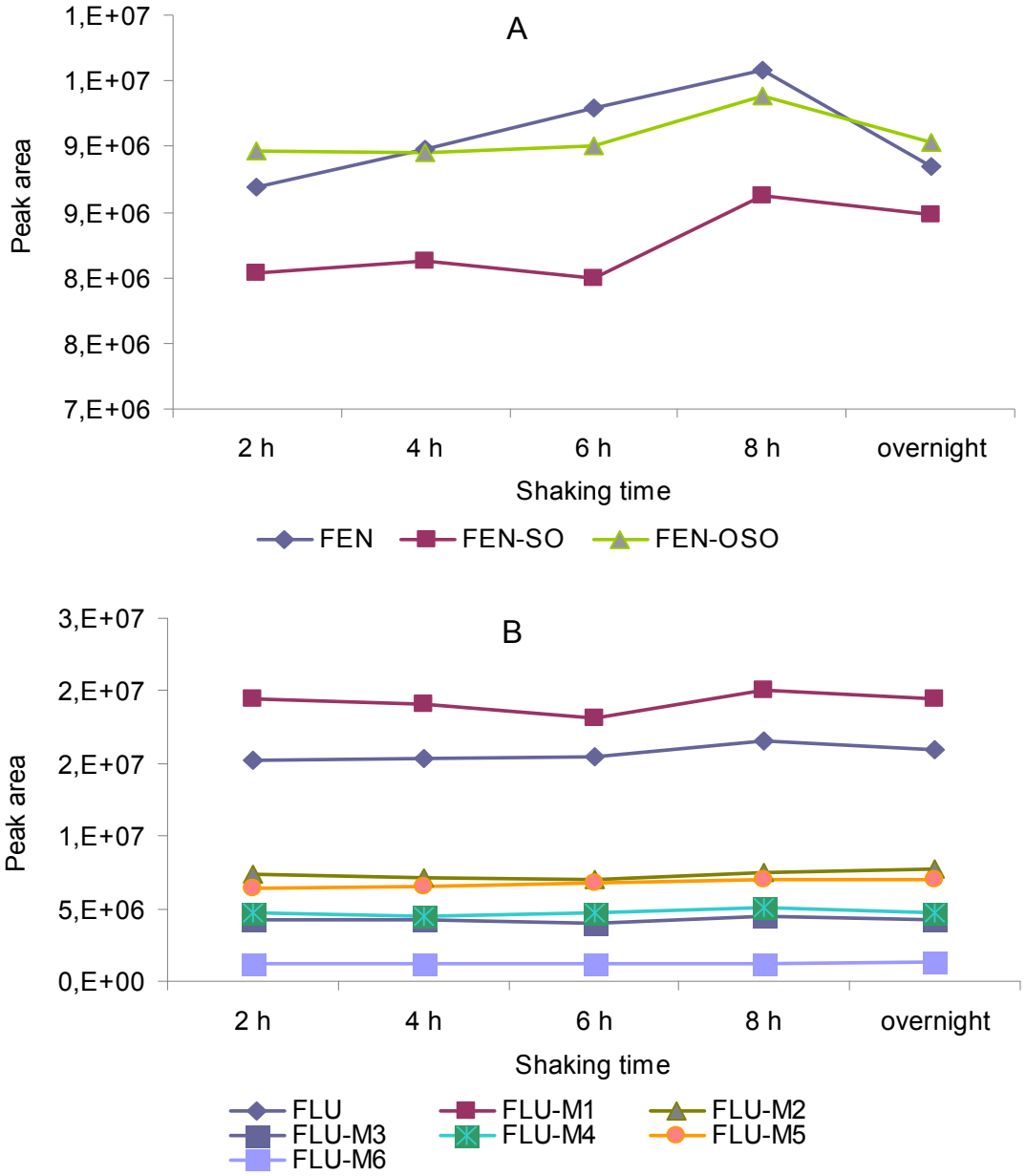


Figure 4.8: Recoveries of A: fenbendazole and B: flubendazole with corresponding metabolites in liquid manure samples extracted with ethyl acetate at pH 9.5 and at different extraction times.

All compounds were extracted with high recoveries ranged from 85-99 %, 78-111 %, 78-100 % and 82-94 % with maximum RSD of 19%. As shown in **Figure 4.9**, this procedure allowed the extraction of target compounds with adequate recoveries. Even though the analysis of benzimidazoles is more complicated in manure samples than biological samples, the obtained recoveries in this work are better than or still comparable with that of previous studies for liquid and solid matrices. Thus, Fletouris et al. (1996) extracted 10 benzimidazole residues including, FEN, FEN-SO, FEN-OH and FEN-OSO from milk samples at pH 10 using ethyl acetate.

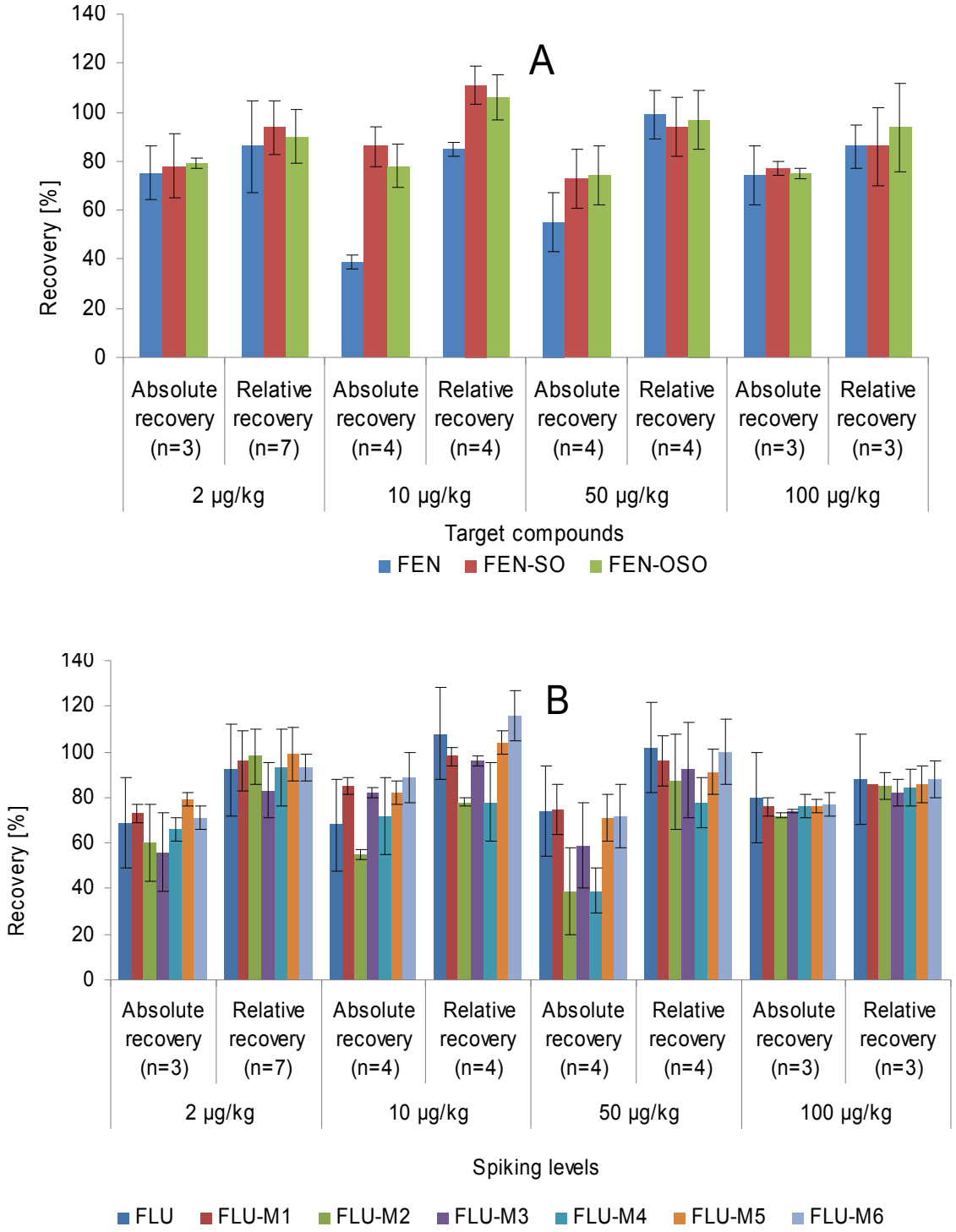


Figure 4.9: Recovery rates of A: fenbendazole and B: flubendazole with corresponding metabolites in liquid manure samples extracted using ethyl acetate at pH 9.5 with different spiking levels. Relative standard deviations are given as error bars.

The extraction efficiency was evaluated over the pH of range 3 to 11.5. The highest recoveries for the majority of investigated benzimidazole residues were given at pH 10. Mean recovery for benzimidazole residues was typically in the range of 79-100 %. A lower recovery in the range of 56-66 % was obtained for FEN.

Aguilera et al. (2012) developed a method for determination of FEN, FEN-SO with some other veterinary drug residues in meat-based baby food and powdered milk-based infant formulae. The extraction procedure based on a buffered quick, easy, cheap, effective, rugged and safe methodology (QuEChERS) using acetonitrile acidified with 1% formic acid (v/v) without any further clean-up step, followed by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC/MS/MS). The obtained recoveries were 123 and 40 % with RSD ≤ 20 % in the meat based food, while in the milk based food recoveries were 117 and 118 % with RSD ≤ 18 % for FEN and FEN-SO, respectively. Moreover, supercritical fluid extraction (SFE) method has been applied for extraction FEN, FEN-SO, FEN-OSO, FLU with 6 other benzimidazole drug residues from animal liver. The obtained recoveries were not quantitative. Mean recovery ranged between 51 and 115 % for the various benzimidazoles. The intra- and inter-assay variations showed RSD values of 10 and 32 %, respectively (Danaher, 2003).

4.1.2.2 Solid-liquid extraction after lyophilization

Alternatively, liquid manure samples were treated by means of lyophilization in order to remove the aqueous phase prior to solvent extract of the manure solids. Recovery experiments were used to determine the efficiency of different organic solvents, e.g., methanol, acetonitrile, acetone and ethyl acetate. The samples were spiked with target compounds at 100 µg/kg fresh manure, extracted in 3 consecutive steps including shaking with 100 mL extracting solvent at 220 rpm overnight and additional rinsing steps with 100 mL and 50 mL shacked again for 60 and 30 min. The obtained extracts were combined, rotary evaporated, cleaned-up and finally analyzed via LC/MS/MS.

Some conclusions can be drawn from those tests. For all solvents used, the obtained recovery ranges of FLU-M1, FLU-M5 and the parent compounds were from 74 % to 103 %. Lowest recoveries ranging from only 40 to 85 % were obtained for FEN-SO, FEN-OSO, FLU-M2, FLU-M3, FLU-M4 and FLU-M6 when acetonitrile, acetone or ethyl acetate was used. Using methanol, however, the recoveries for all the target compounds ranged from 96 to 107 % (**Figure 4.10)**.

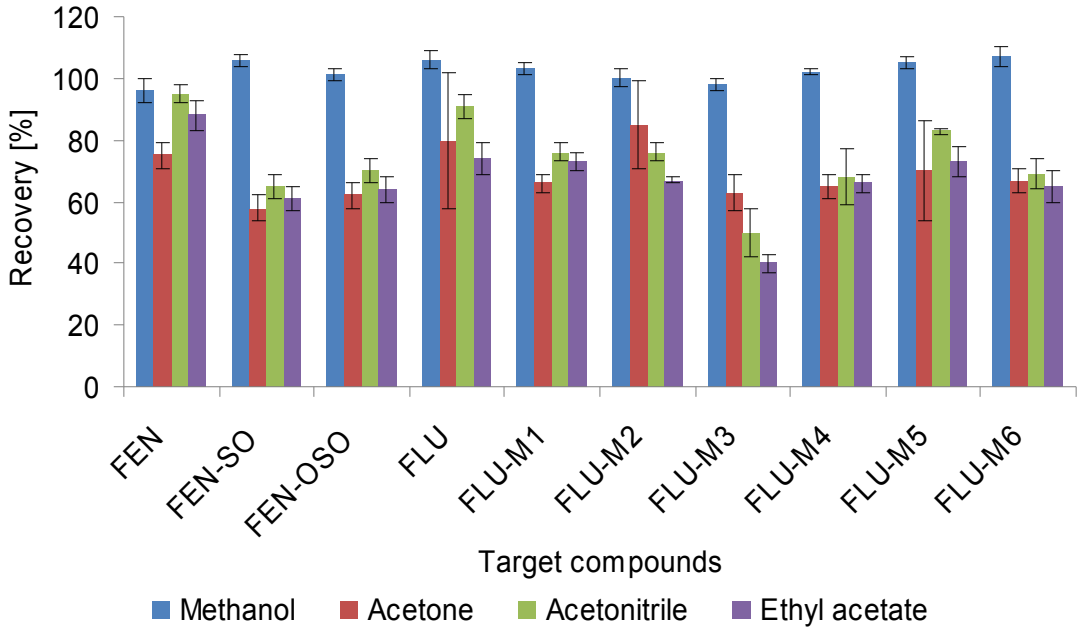


Figure 4.10: Recovery rates of fenbendazole and flubendazole with corresponding metabolites extracted from lyophilized pig manure samples with different solvents.

It was apparent that methanol was the most efficient solvent to extract these compounds from lyophilized manure at original pH. This is due to its wetting power and ability to penetrate and raising the contact with dried manure particles more than the other solvents with lower polarity**.** These results also can be supported by the data published in the literature. Danaher et al. (2007) reported that polar solvents such as methanol and acetonitrile have high efficiency to extract the target compounds from biological and food samples without any need of pH adjustment. Blanchflower et al. (1994) determined FEN and FEN-SO in liver and muscle samples using LC-MS. The samples were homogenized with water, sonicated with methanol and centrifuged. The supernatants were washed with light petroleum and re-extracted with diethyl ether/ethyl acetate. The extracts were evaporated to dryness, dissolved in the mobile phase and injected into the LC-MS system. The mean recoveries were 91% for FEN and 86% for FEN-SO. In another study, approximately 100 % of spiked FEN in milk samples were extracted with acetonitrile over the pH range 2-9, indicating pH adjustment of milk samples was unnecessary when acetonitrile was used as the extractant solvent. This method was optimized for FEN only (Fletouris et al., 1994). Sørensen and Hansen (1998) extracted FEN, FEN-SO and FEN-OSO spiked in trout muscle and skin tissues at 5-150 µg/kg with acetonitrile at neutral pH. The extracts were concentrated and cleaned up by solid phase extraction on C_{18} and CN cartridges where the mean recoveries in muscle were 88, 94 and 92 %, respectively. The corresponding mean recoveries in skin tissue were 88, 81 and 86 % at a level of 10-100 µg/kg. The mean relative

standard deviation was 9 % at a level of 5 µg/kg, 6 % at a level of 10-100 µg/kg and 2 % at a level of 150 µg/kg. Marti et al. (1990) extracted 8 benzimidazole residues including, FEN, FLU and FEN-SO from muscle, liver and kidney tissues using acetonitrile. Recovery of benzimidazole residues was > 65 % for all residues with the exception of FEN-SO which gave a lower recovery of 45 and 39 % for liver and kidney, respectively.

The results of the proposed extraction procedure are similar or better than the methods described for other groups of VMP developed by different research groups. For examples, Pfeifer et al. (2002) extracted trimethoprim with 4 sulfonamides from manure samples using ethyl acetate at pH 5.2 at recovery range from 77 to 91 %. In another study described by Haller et al. (2002) trimethoprim with 7 sulfonamides were extracted from manure samples using ethyl acetate at pH 9 adjusted by adding potassium hydroxide (KOH). The recovery rates from spiked manure slurry samples spiked at 1 mg/kg were 51 to 89 % with RSD < 20 %. In the method developed by Hamscher group (2002) to extract tetracycline group from manure sample at higher concentration levels, e.g., 0.2 to 1 mg/kg. The recovery ranges were 87 to 100 %, 82 to 104 % and 94 to 127 % for oxytetracycline, tetracycline and chlortetracycline, respectively with RSD ≤ 9 %.

Otherwise, it should be noted that a parallel increase of the co-extracts was also observed due to this non selective extraction procedures. For this reason, it was necessary to add SPE (SDB1) as another clean-up step combined with SEC which has been already used as single clean-up step during the preliminary work. After identification of the optimum extraction conditions, series of fortification experiments were conducted to evaluate the performance of the optimized method at different spiking levels covering the environmentally relative concentrations, e.g., 4, 10, 50 and 100 µg/kg. The range of percentage recoveries for all the target compounds was 76 to 106 % with RSD < 14. The mean recoveries are given in **Figure 4.11.** The analytical scheme of the optimized methods for benzimidazoles analysis in pig manure samples directly extracted using ethyl acetate or after lyophilization using methanol are summarized in **Figure 4.12**.

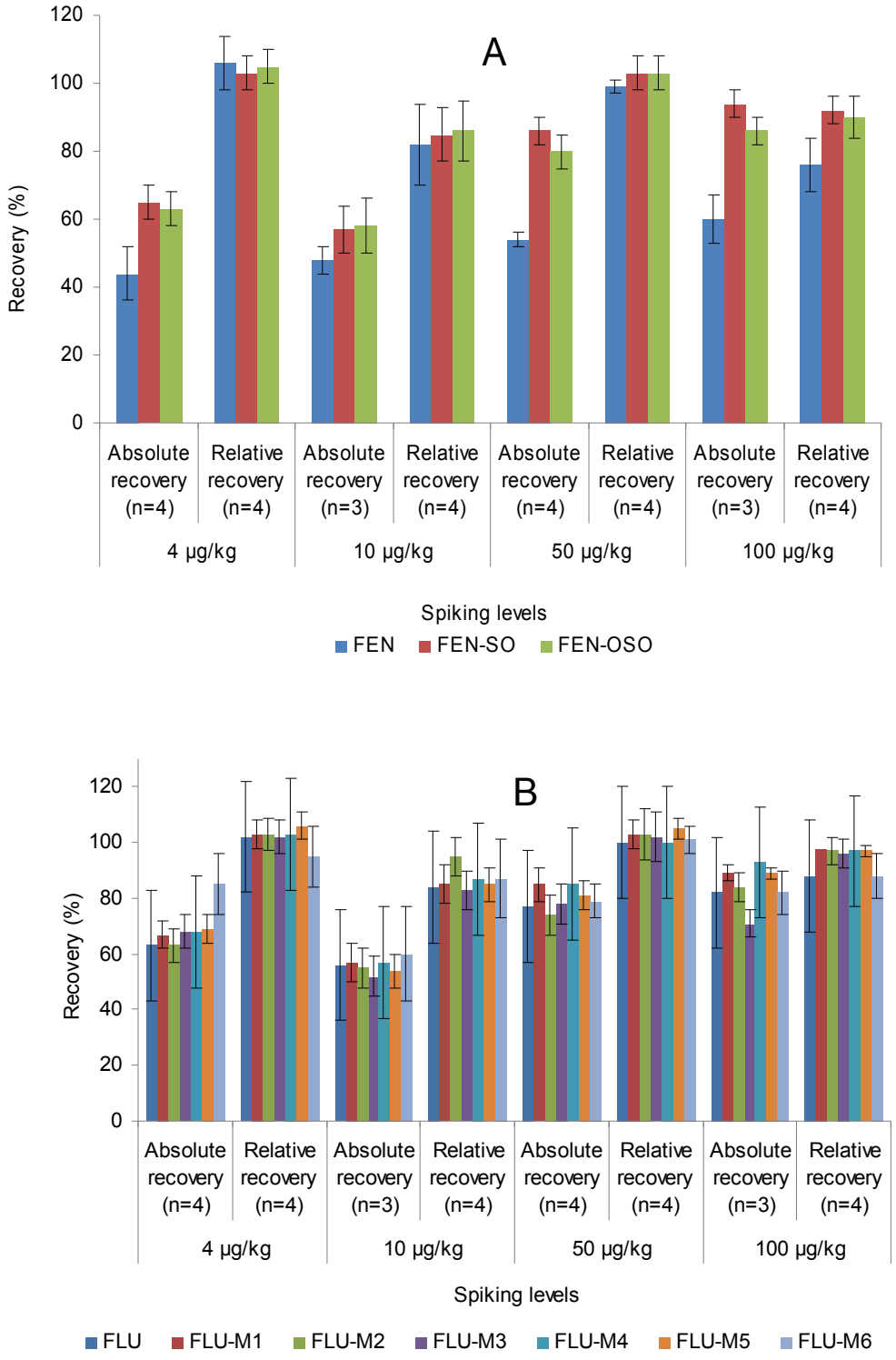


Figure 4.11: Recovery rates of A: fenbendazole and B: flubendazole with corresponding metabolites extracted from lyophilized manure samples using methanol with different spiking levels at original pH.

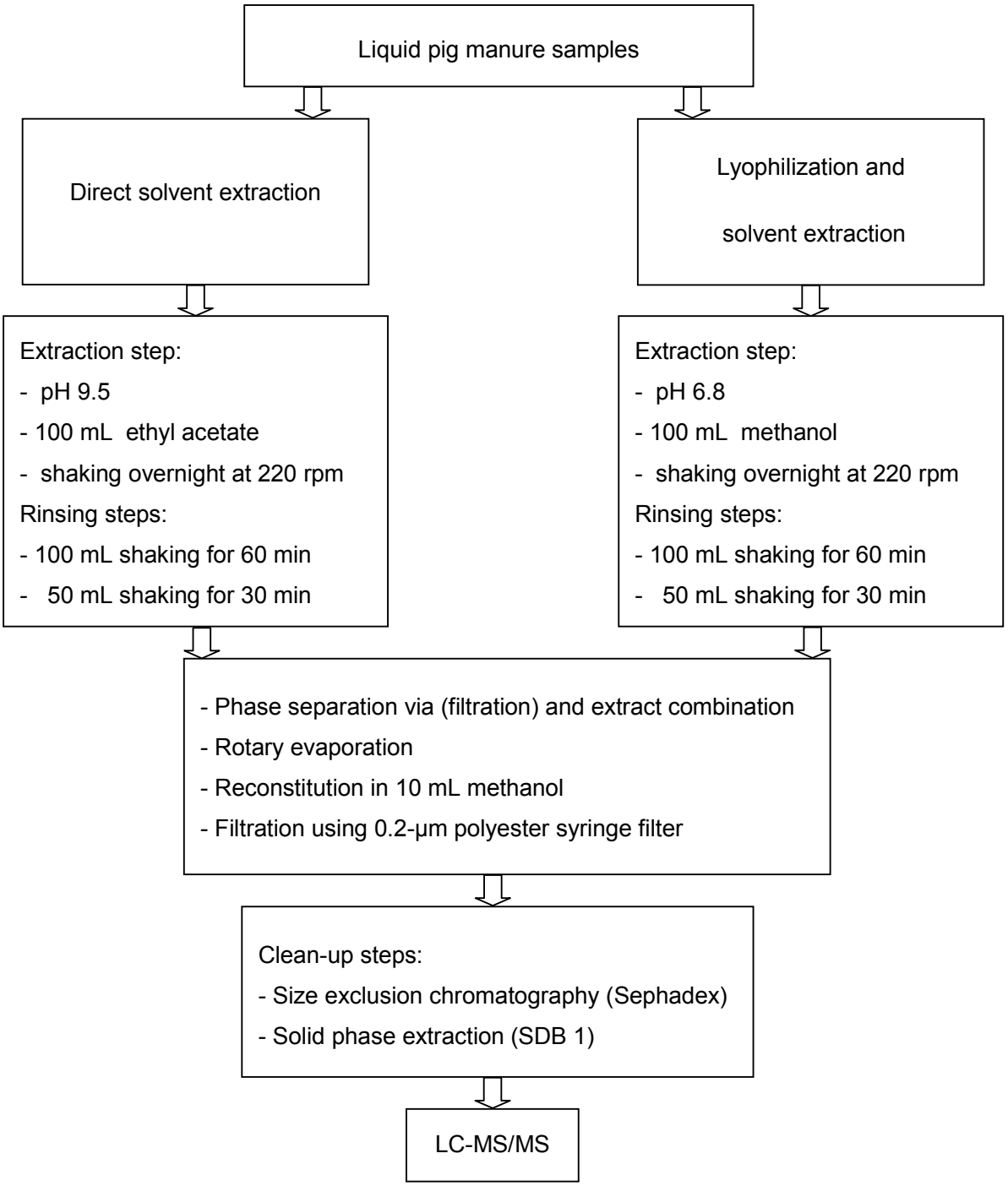


Figure 4.12: Analytical scheme of the optimized methods for the target benzimidazole analysis in pig manure samples.

4.1.2.3 Ultrasound-assisted solvent extraction

Furthermore, the efficiency of an ultrasound-assisted solvent extraction (USE) was checked to release the target benzimidazoles out of liquid pig manure samples. USE is a technique which has been used in a wide range of environmental samples as simple, rapid and low-cost method (Babic et al., 1998; Aydin et al., 2007).

To apply USE technique, liquid manure samples were differently prepared. A general scheme of the developed analytical method is illustrated in **Figure 4.13**. First, samples were spiked at 100 µg/kg and then directly extracted using ultrasound- assisted extraction at pH 4.4.

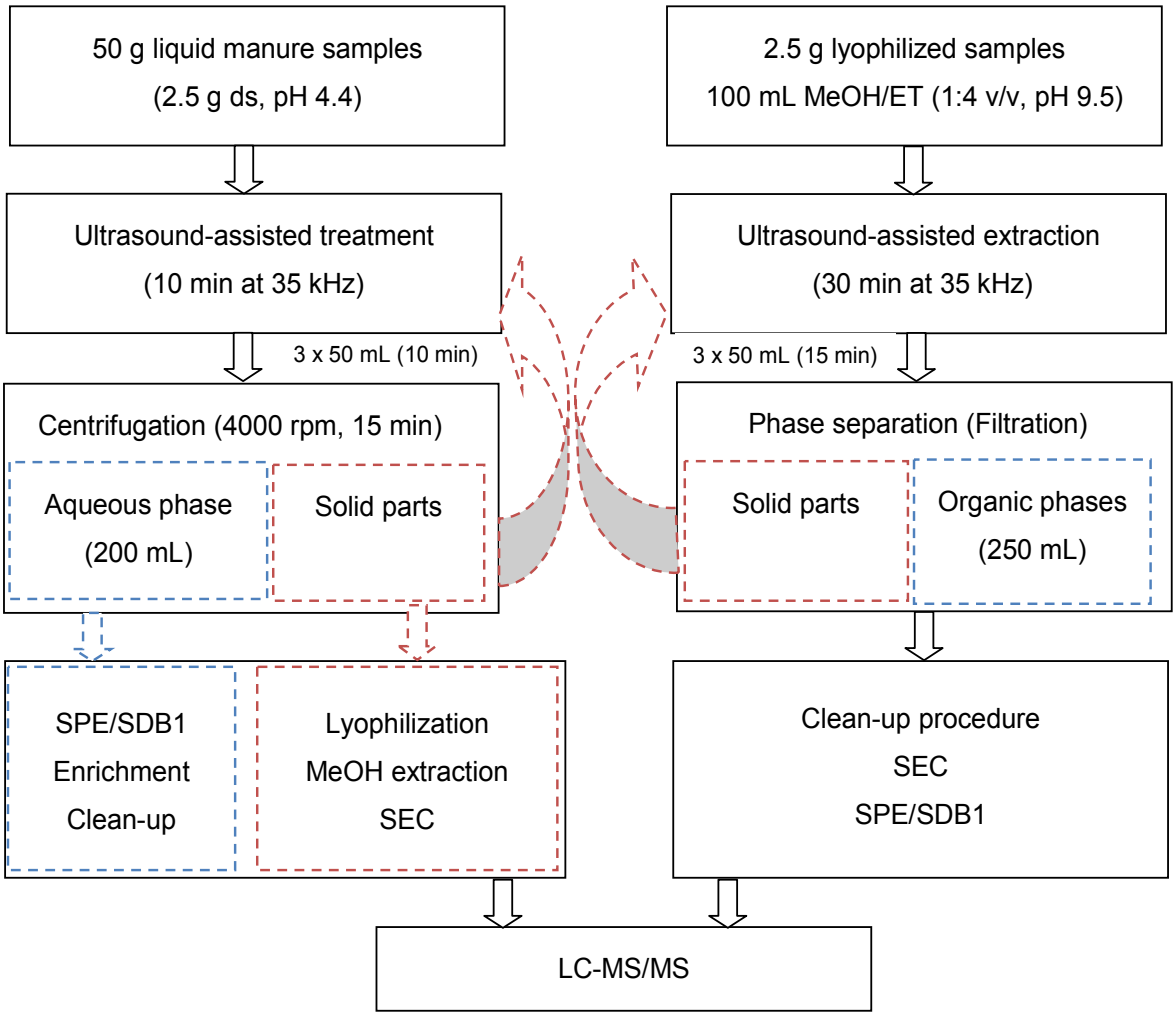


Figure 4.13: Analytical scheme of the optimized methods for target benzimidazoles analysis in pig manure samples using ultrasound-assisted solvent extraction method.

The liquid phases were separated by centrifugation and then cleaned up and concentrated by solid phase extraction using SDB1 cartridges. Recoveries in the aqueous varied, e.g., from 15 % for FEN to 53 % for FEN-SO and 27 % for FLU to 69 % for FLU-M6 due to the physico-chemical properties of the parent compounds and the metabolites (**Figure 4.14**). In contrast, 73 % of FEN and 22 % FEN-SO as well as 64 % of FLU and 10 % FLU-M6 were recovered in the solid phases which were extracted by means of methanol shaking overnight. These results reflected the physico-chemical properties of the target compounds. Hence, the more hydrophobic parent compounds FEN and FLU as well as FEN-OSO were sorbet to the manure solids while the more polar metabolites mainly occur in the aqueous phases.

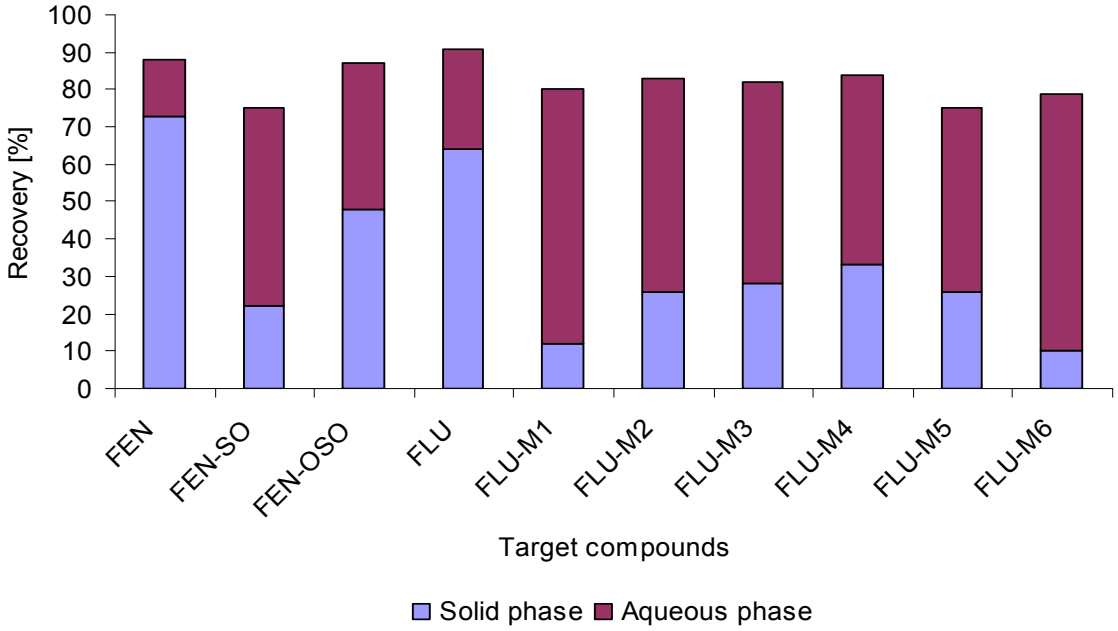


Figure 4.14: Recovery rates of fenbendazole and flubendazole with corresponding metabolites spiked into liquid pig manure samples at 100 µg/kg.

Within the second approach, the liquid manure samples were initially lyophilized. The manure solids were then treated with different solvents. Here, methanol was an efficient solvent to extract all the target compounds with recovery rates > 90 % at original pH. In contrary, very low recoveries were achieved for the majority of the target compounds when the solids were ethyl acetate extracted. With exception of FEN, FLU, FLU-M1 and FLU-M5, the obtained recoveries ranged from 40 to 65 %. It is attributed to the decrease of the penetration power of ethyl acetate to solid manure particles due its hydrophobicity.

An additional solvent mixture consisted of methanol and ethyl acetate (1:4, v/v), therefore, was used to extract the target compounds from lyophilized manure samples at alkaline pH using USE technique. Initially, experiments were performed in order to establish the number

of extraction and rinsing cycles as the most critical step after solvent selection in USE method. In the extraction cycle, lyophilized manure samples were ultrasonicated with 100 mL solvent mixture for 30 min where 59 to 66 % of all target compounds were extracted. The first rinsing cycle was important to reach the acceptable recovery range of > 70 %, where 26 to 31 % of spiked analytes were recovered in this step via ultrasonication with 50 mL solvent for 15 min (**Figure 4.15**).

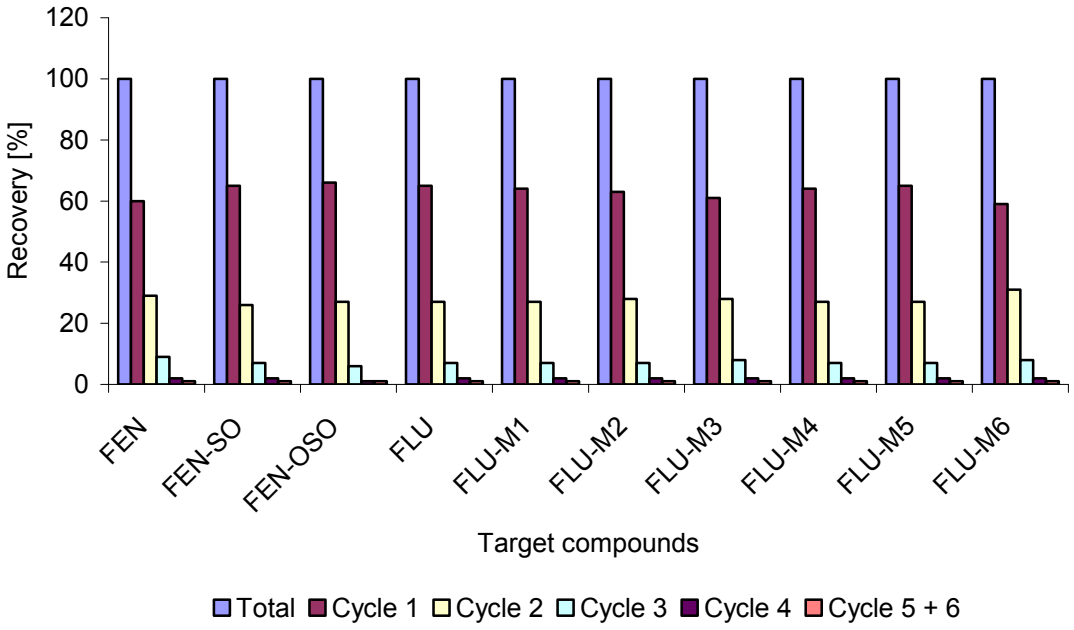


Figure 4.15: Recoveries of fenbendazole and flubendazole with corresponding metabolites from lyophilized liquid manure samples after 1 extraction and 5 rinsing cycles using methanol/ethyl acetate mixture (1:4; v/v; pH 9.5).

In the two next rinsing cycles, only < 11 % of the analytes was recovered. For analytical quality aspects, however, these two rinsing cycles were included in the final analytical procedure. The optimum extraction procedure was established as one extraction step using 100 mL ethyl acetate/methanol mixture (4:1 v/v) sonicated for 30 min followed by 3 rinsing steps, each one was sonicated for 15 min with 50 mL solvent mixture. This method was evaluated based on recovery experiments at different spiking levels of the target compounds, i.e., 4, 50 and 100 µg/kg. Recoveries were between 94 and 109 % with RSD < 12 %. These results indicated that combination of ethyl acetate and methanol at alkaline pH resulted in optimum recoveries for all target compounds. Lowest recovery 67 % with RSD ≤ 14 % was found for FEN at 50 µg/kg (**Figure 4.16**).

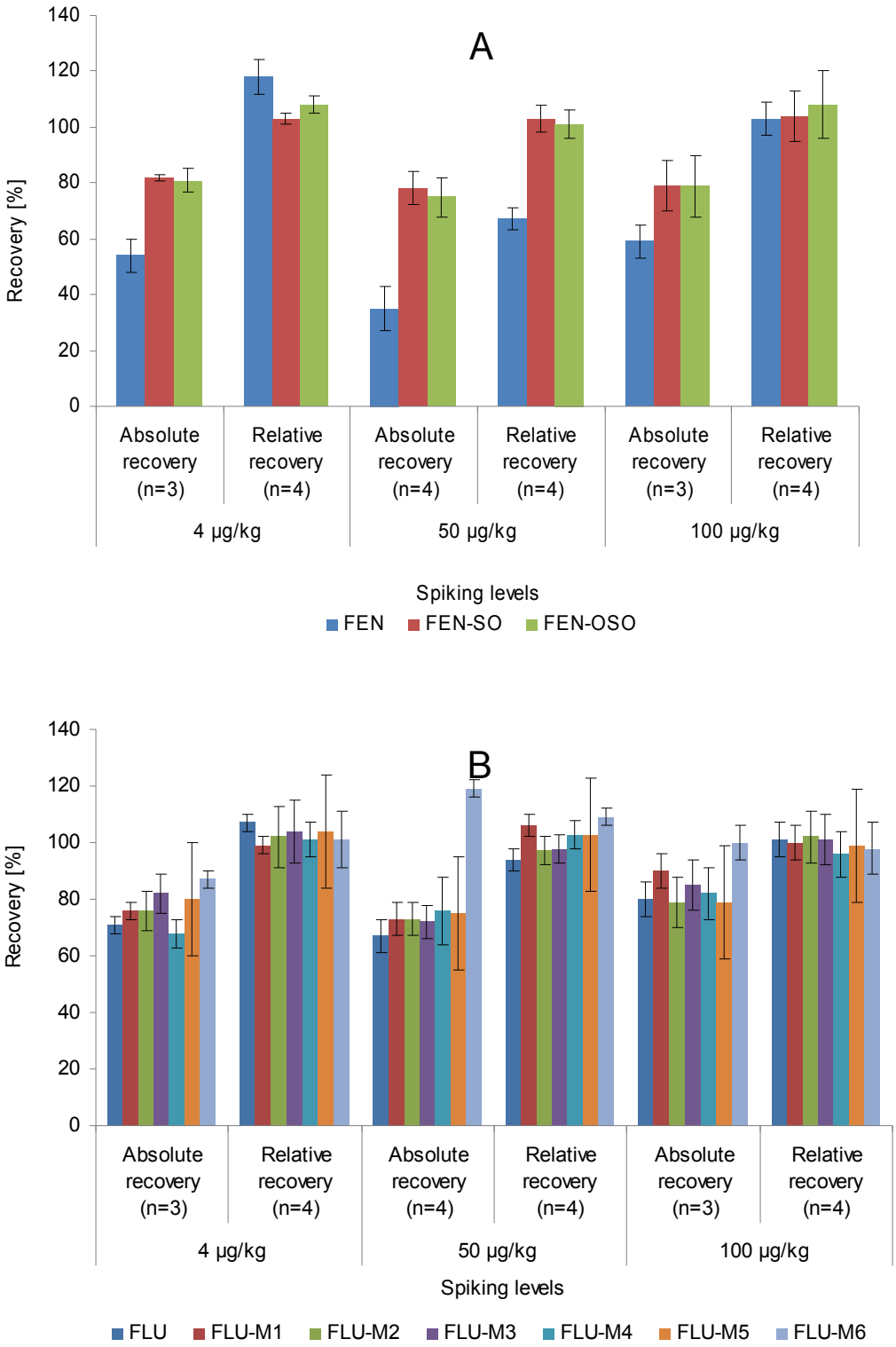


Figure 4.16: Recoveries of A: fenbendazole and B: flubendazole with corresponding metabolites of spiked lyophilized manure samples extracted with methanol/ethyl acetate mixture (1:4, v/v) at pH 9.5 via ultrasound-assisted extraction.

4.1.3 Soil and manured soil samples

4.1.3.1 Direct solvent extraction

Soil samples

The extraction procedures checked for sand and clay soil samples were optimized with regard to several relevant parameters, e.g., solvent types, number of rinsing cycles after extraction step and pH as well as the extraction time. First, different solvents were tested to select the best solvent to achieve a high extraction efficiency of the target compounds. On the basis of the procedure developed by Kreuzig et al. (2007), the initial experiments were conducted using methanol without pH adjustment.

50 g sand soil samples were spiked with target compounds at 100 µg/kg, mixed well and then left at room temperature about 1 h to evaporate the spiking solvent. Subsequently, the spiked samples were shaken on a horizontal shaker with 100 mL at 250 rpm overnight. The extracted soil samples were rinsed twice with 100 mL solvent and shaken again for 2 h and 1 h. Several solvents with different polarity and their combinations, including methanol, acetonitrile, acetone, ethyl acetate, methanol/ethyl acetate (1:1 or 1:4) and acetone/ethyl acetate (1:4) were tested. All the solvents and solvent combinations recovered most of the target compounds with acceptable recovery rates. **Table 4.2** and **4.3** show the mean recoveries of target compound spiked in sand and clay soil samples at 100 µg/kg. Thus, the recovery rates of FEN, FEN-SO, FEN-OSO, FLU, FLU-M5 and FLU-M6 analytes were in the range 56 to 114 % for methanol, acetone, acetonitrile, mixtures of methanol/ethyl acetate at different ratios (1:1 and 1:4, v/v) and mixture of acetone/ethyl acetate (1:4).

Table 4.2: Recovery rates of fenbendazole and flubendazole with corresponding metabolites from sand soil spiked at 100 µg/kg using different solvents (n=4) (Recovery % = concentration spiked before extraction / concentration spiked before extraction).

Target compounds	Mean recoveries [%] ± RSD [%]										
	MeOH	ACE	ACN	ET	ET[a]	MeOH/ ET [b1] (1:1, v/v)	MeOH/ ET [b2] (1:4, v/v)	MeOH/ ET [c] (1:4, v/v)	MeOH/ ET [d] (1:4, v/v)	ACE/ET (1:4, v/v)	ACE/ ET [e] (1:4, v/v)
FEN	80 ± 3	114 ± 8	67 ± 4	98 ± 26	94 ± 6	88 ± 7	110 ± 4	120 ± 2	114 ± 8	91 ± 12	108 ± 20
FEN-SO	75 ± 1	60 ± 6	87 ± 6	88 ± 9	93 ± 8	74 ± 5	75 ± 6	100 ± 6	83 ± 5	63 ± 3	91 ± 12
FEN-OSO	78 ± 1	76 ± 5	87 ± 7	86 ± 11	95 ± 7	85 ± 6	96 ± 5	98 ± 5	99 ± 7	91 ± 1	91 ± 14
FLU	81 ± 1	73 ± 7	85 ± 7	83 ± 19	95 ± 8	90 ± 5	102 ± 3	100 ± 3	108 ± 8	98 ± 4	96 ± 19
FLU-M1	83 ± 1	69 ± 3	83 ± 7	102 ± 22	91 ± 5	88 ± 3	94 ± 5	90 ± 6	89 ± 8	80 ± 0	100 ± 4
FLU-M2	26 ± 7	13 ± 7	13 ± 19	36 ± 31	97 ± 14	29 ± 9	16 ± 11	98 ± 8	10 ± 10	4 ± 30	102 ± 9
FLU-M3	18 ± 28	9 ± 53	30 ± 20	12 ± 36	91 ± 14	35 ± 3	11 ± 16	93 ± 6	5 ± 70	9 ± 21	90 ± 4
FLU-M4	26 ± 3	10 ± 6	26 ± 10	11 ± 39	91 ± 12	41 ± 5	12 ± 9	96 ± 4	3 ± 17	14 ± 21	90 ± 4
FLU-M5	72 ± 3	56 ± 11	66 ± 8	87 ± 4	94 ± 7	76 ± 5	77 ± 6	92 ± 6	68 ± 7	63 ± 1	93 ± 8
FLU-M6	81 ±1	71 ± 10	74 ± 7	100 ± 9	94 ± 6	91 ± 4	88 ± 7	95 ± 6	95 ± 6	88 ± 5	96 ± 4

ACE: acetone; MeOH: methanol; ACN: acetonitrile; ET: ethyl acetate; (a) 35 mL ammonium chloride buffer were added; (b1, 2) solvent mixture with different ratios; (c): 5 mL ammonium chloride buffer were added (d): 0.5 mL formic acid was added; (e): 5 mL buffer (pH 9.5), n= 3 except FEN was 2.

Table 4.3: Recovery rates of fenbendazole and flubendazole with corresponding metabolites from clay soil spiked at 100 µg/kg using different solvents (Recovery = concentration spiked before extraction/concentration spiked after extraction).

Target compounds	Mean recoveries ± RSD [%]			
	MeOH (n=3)	ACN (n=4)	ET[a] (n=3)	MeOH/ET [b] (n=4) (1:4, v/v)
FEN	78 ± 2	84 ± 5	94 ± 5	100 ± 3
FEN-SO	58 ± 4	47 ± 4	98 ± 8	100 ± 3
FEN-OSO	71 ± 4	77 ± 3	90 ± 11	94 ± 5
FLU	77 ± 6	86 ± 3	98 ± 7	97 ± 2
FLU-M1	83 ± 4	79 ± 3	99 ± 7	85 ± 8
FLU-M2	40 ± 5	17 ± 11	91 ± 8	93 ± 3
FLU-M3	25 ± 8	5 ± 38	77 ± 82	89 ± 8
FLU-M4	23 ± 6	8 ± 15	97 ± 5	91 ± 4
FLU-M5	67 ± 6	60 ± 3	96 ± 8	95 ± 1
FLU-M6	74 ± 3	72 ± 3	86 ± 13	87 ± 7

MeOH: methanol; ACN: acetonitrile; ET: ethyl acetate;

[a]: 35 mL ammonium chloride buffer were added; [b]: 5 mL ammonium chloride buffer were added.

However, the obtained recoveries for FLU-M2, FLU-M3 and FLU-M4 were below 35 %. The target compounds were extracted with recovery rates ranged from 68 to 114 % by a mixture of methanol/ethyl acetate (1:4 v/v) acidified with 0.5 mL formic acid. For FLU-M3 and FLU-M4, however, recoveries were below 5 %. At alkaline pH, using 35 mL ammonium chloride buffer, ethyl acetate was an efficient solvent to extract the target compounds with recovery ranges more > 90 % with RSD < 14 % for the sand soil. However, this procedure was not efficient for FLU-M3 in case of clay soil. Non repeatable results were obtained for this target compound reflected by recoveries of 77 % and RSD of 82 %. This may be attributed to the clay content of this soil that may decrease the contact of the solvent with soil particles even with presence of big volume of buffer solution (35 mL). Contrary, > 85 % of the spiked target compounds were recovered from these two differently textured soils by solvent combination, e.g., methanol/ethyl acetate (1:4, v/v) or acetone/ethyl acetate (1:4, v/v) at alkaline pH adjusted by adding 5 mL ammonium chloride buffer.

Once the optimum extraction conditions were identified, the other parameters such as number of rising cycles and shaking time during the extraction step were tested. The results showed that two rinsing cycles after the extraction step were necessary to achieve the acceptable recovery ranges. The recoveries by the single extraction step were ≤ 62% for all of the target compounds. Approximately 40 % of the spiked target compounds were recovered by the second and third rinsing steps (**Figure 4.17**).

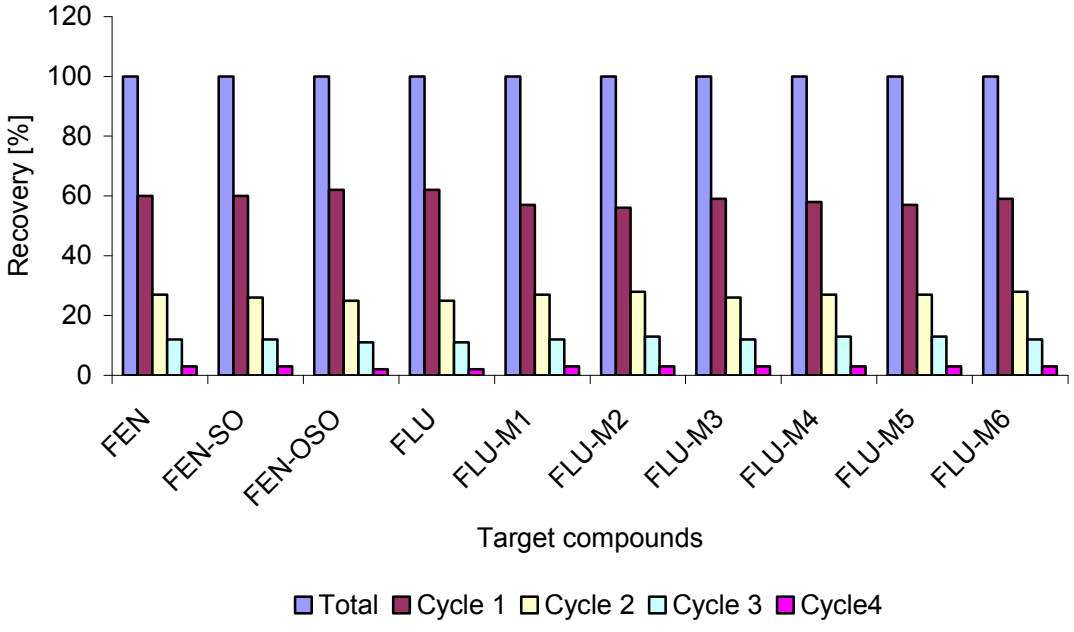


Figure 4.17: Recovery rates of fenbendazole and flubendazole with corresponding metabolites obtained after 1 extraction and 3 rinsing cycles from sand soil samples spiked at 100 µg/kg using methanol/ethyl acetate (1:4, v/v) at alkaline pH.

It should be noted that the fortification experiments were conducted at best-case extraction conditions, where the target compounds were extracted directly after the fortification and distribution in the sample matrices to avoid any aging of the test substances. Therefore, there was no big difference between the obtained recoveries after 2, 6 and 8 h or shaking overnight **(Figure 4.18)**. Considering the aging effect on the extractability of the target compounds, therefore, shaking overnight combined with two rinsing steps were chosen to successfully extract all target compounds from soil samples at alkaline pH. These results confirmed that a combination of ethyl acetate with another polar solvent such as methanol or acetone at alkaline pH improved the extraction efficiencies.

The efficiency of the optimized extraction procedure to extract the target compounds from two differently textured soils was also examined at different spiking levels. The average recovery rates of all target compounds were in the range of 83 to 120 % with RSD ranging from 3 to 15 % at different spiking levels, i.e., 2, 4, 40 and 100 µg/kg in sand soil **Figure 4.19**. At spiking level of 4 and 2 µg/kg, only FEN and FLU-M2 showed recoveries of 56 and 70% with RSD < 13 and 21 %, respectively. This was attributed to spiking levels close to the method limit of quantitation (MQL). In clay soil, all target compounds were efficiently extracted with high recoveries rates ranging from 83 to 100 % with RSD < 16 % at different spiking levels, i.e., 4, 40 and 100 µg/kg. The mean recoveries of all target compounds are shown in **Figure 4.20**.

Taken into account that the European commission Decision 2002/657/EG (EC, 2002) for identification, quantitation and confirmation of organic pollutants in live animals and animal products accepts recoveries ranged from 50 to 120 %, 70 to 110 % and 80 to 110 % at spiking concentration < 1, < 10 and > 10 µg/kg respectively, while the acceptable range in EPA is 70 to 120 %. The results show that this method allowed the extraction of all the analytes at acceptable recovery ranges (Yokley et al., 2000).

The obtained results were in agreement with those of other studies. For example, the obtained recoveries were 96 % for FEN and 93 % for FLU from soil sample extracted with ethyl acetate at pH 5.2. Only 58 % of FEN and 78 % of FLU in soil samples were recovered using methanol as described by Kreuzig et al. (2007). Dowiling et al., (2005) developed a method for the analyses of 12 benzimidazoles, e.g., FEN, FEN-SO, FEN-OSO, FLU and FLU-M2 in bovine liver using ethyl acetate at alkaline pH. Liver samples were extracted with mean recovery between 35 and 85 % with RSD < 25%. Moreover, the efficiency of the proposed extraction procedure to extract the target compounds from soil samples is similar or better than the methods described for other groups of VMP developed by different research groups. In a method developed for simultaneous analysis of a mixture of tetracyclines, e.g., chlorotetracycline and oxytetracycline, macrolides, e.g., tylosin and erythromycin and sulfonamide, e.g., sulfadiazine from agricultural soils, these compounds

were extracted using methanol at pH 4.7 at recovery rates of 50-80 % for tetracyclines and sulfonamide compounds and 60 to 100 % for the macrolides as described by Jacobsen et al., (2004). Hamscher et al. (2002) extracted oxytetracycline, tetracycline, chlortetracycline and tylosin form soil sample at acidic pH using citrate buffer at pH 4.7. At spiking levels from 5 to 100 µg/kg, the recovery rates ranged from 66 to 86 %, 33 to 46 %, 57 to 76 % and 60 to 66 % with RSD < 20, 29, 21 and 15 % for oxytetracycline, tetracycline, chlorotetracycline and tylosin, respectively.

Manured soil samples

The procedure optimized for extraction of target compounds from soil samples were directly used to extract these compounds from manured soil samples as well. The spiked samples with target compounds at 100 µg/kg were extracted using methanol/ethyl acetate (1:4, v/v) at alkaline pH. The combined extracts were rotary evaporated, cleaned up by SEC and SPE (SDB1) and finally analyzed by LC/MS/MS. The recovery values ranged from 84 to 94 % and 88 to 103 % for all target compounds in manured sand and clay soil samples, respectively. Efficiency of the extraction procedure was also evaluated at different spiking levels. In manured sand soil, high recoveries rates with acceptable RSD% were obtained for all target compounds. The mean recoveries values ranged from 83 to 110 % with RSD ≤ 16 % for all the target compounds spiked at 2, 10, 50 and 100 µg/kg (**Figure 4.21)**.

The efficiency of the extraction procedure was tested in manured clay soil only at 2 and 100 µg/kg spiking levels. High recovery rates were also achieved for all target compounds. At low spiking level, the mean recovery rates were 81 to 91 with RSD ≤ 7 %, while at high spiking levels recovery rates were ranged from 88 to 100 % with RSD ≤ 10 % (**Figure 4.22**).

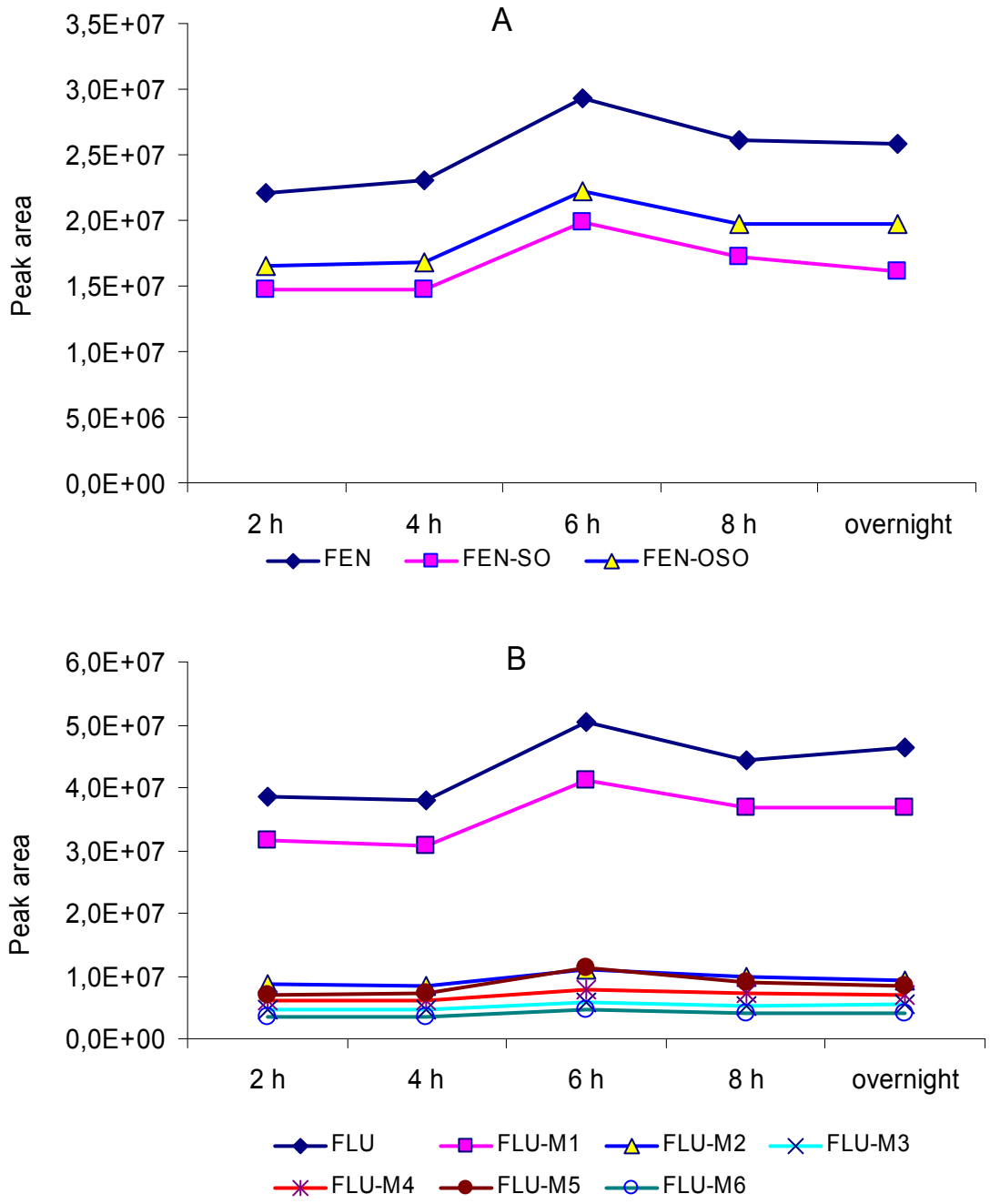


Figure 4.18: Recovery rates of A: fenbendazole and B: flubendazole with their corresponding metabolites in dependency the extraction time in sand soil samples spiked at 100 µg/kg using methanol/ethyl acetate (1:4, v/v) at alkaline pH.

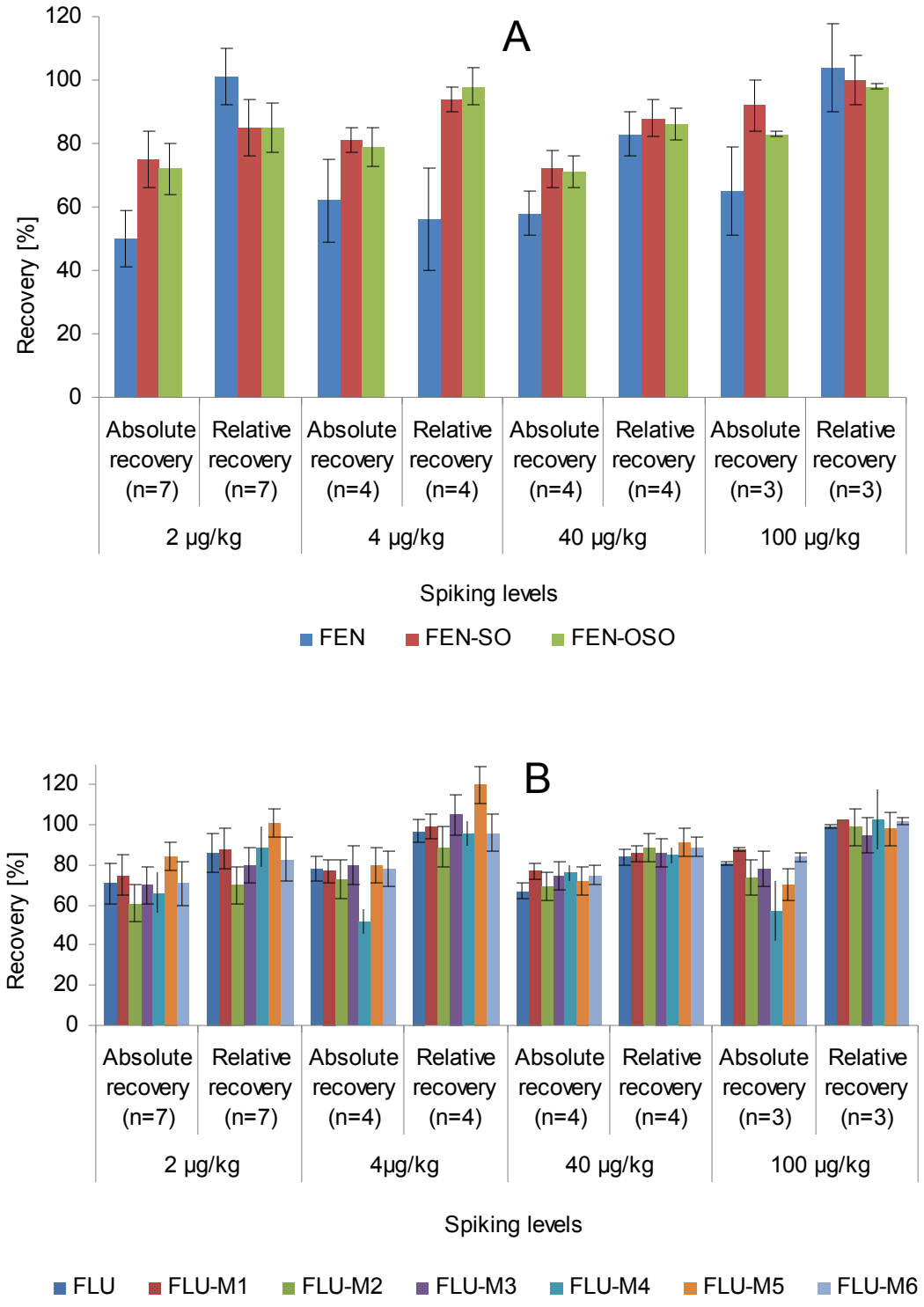


Figure 4.19: Recovery rates of A: fenbendazole and B: flubendazole with corresponding metabolites spiked to sand soil samples and extracted with methanol/ethyl acetate mixture (1:4, v/v) at alkaline pH.

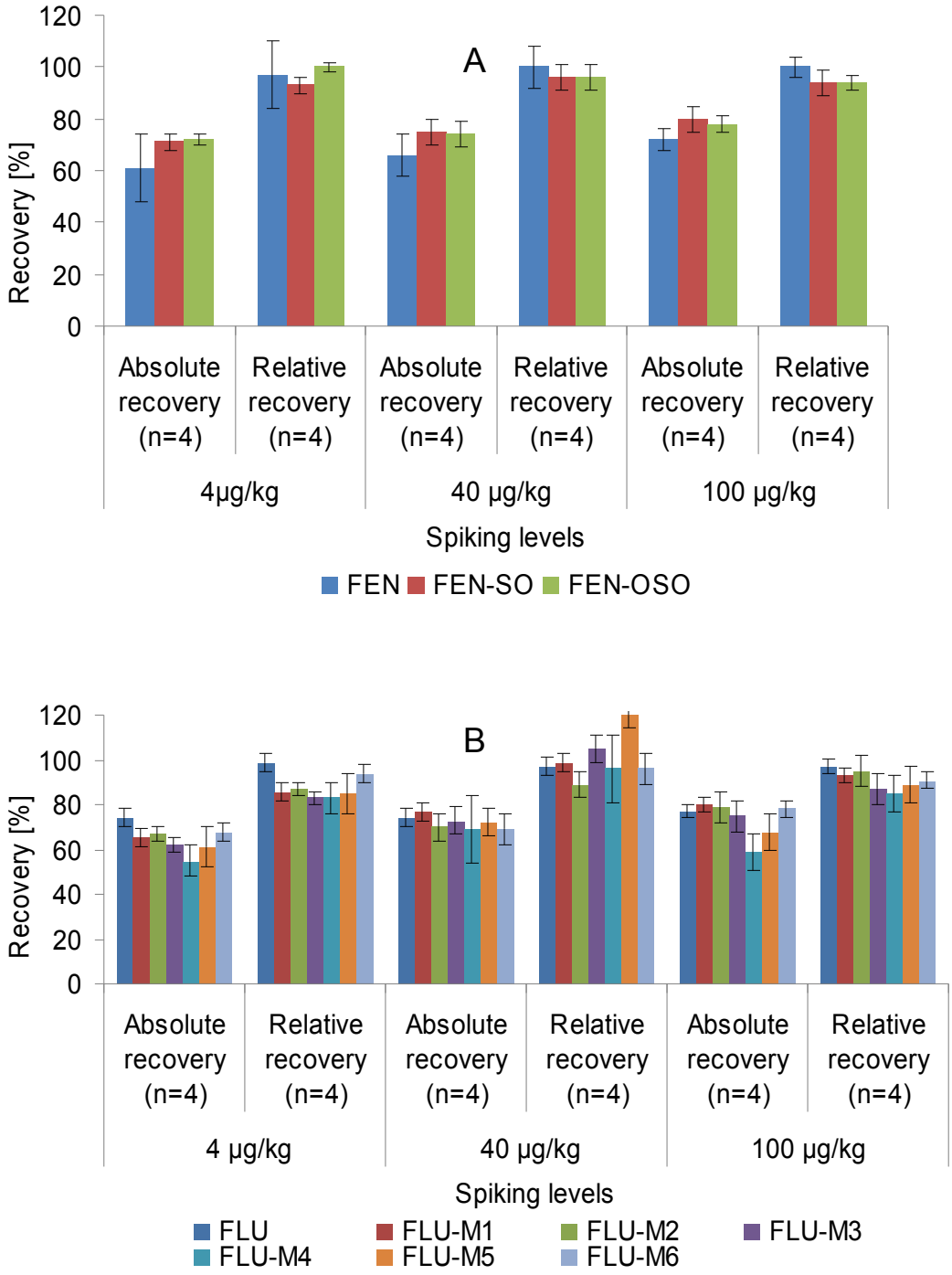


Figure 4.20: Recovery rates of A: fenbendazole and B: flubendazole with corresponding metabolites spiked to clay soil samples and extracted with methanol/ethyl acetate mixture (1:4, v/v) at alkaline pH.

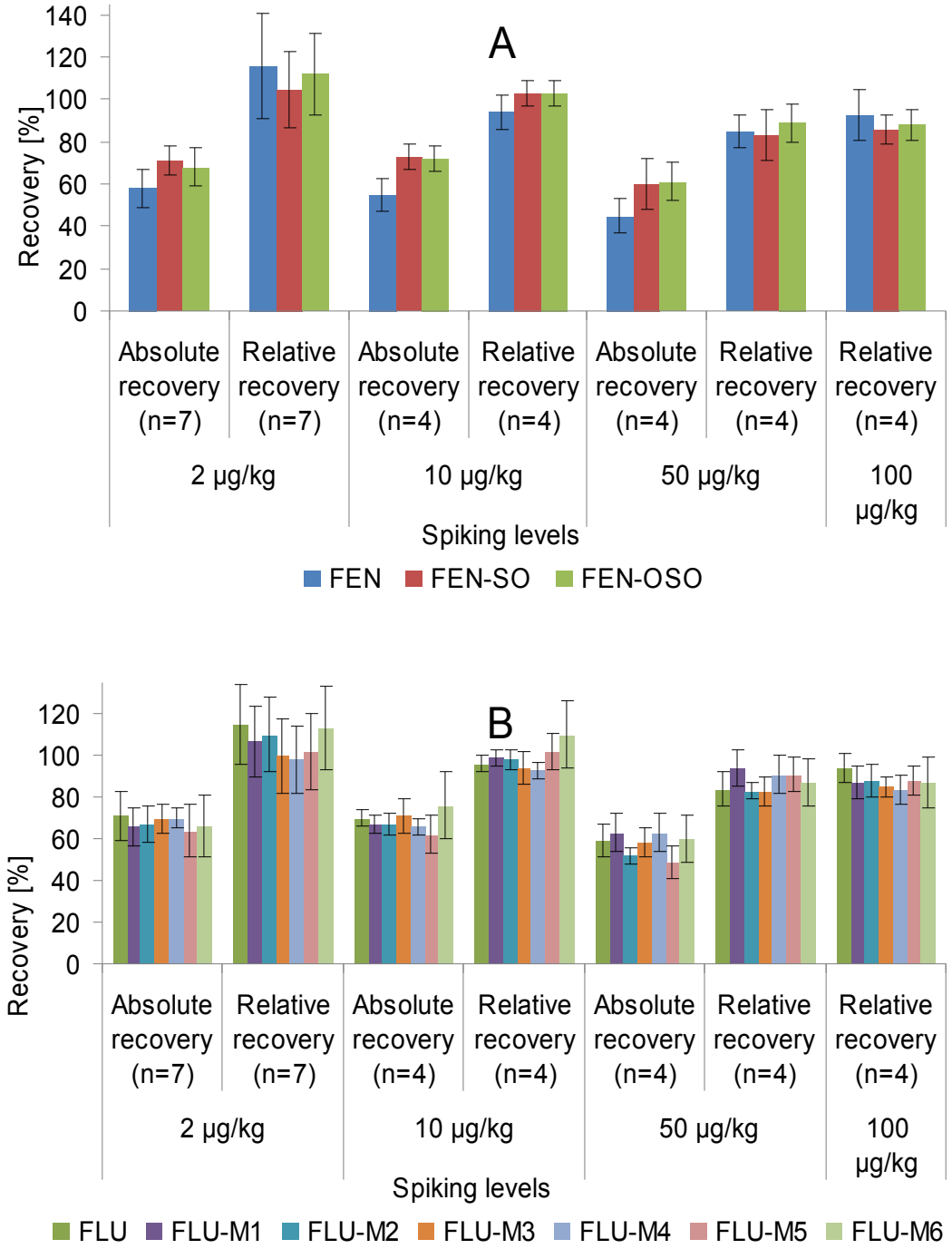


Figure 4.21: Recovery rates of A: fenbendazole and B: flubendazole with corresponding metabolites spiked in manured sandy soil samples at different spiking levels and extracted with methanol/ ethyl acetate mixture (1:4, v/v) at alkaline pH.

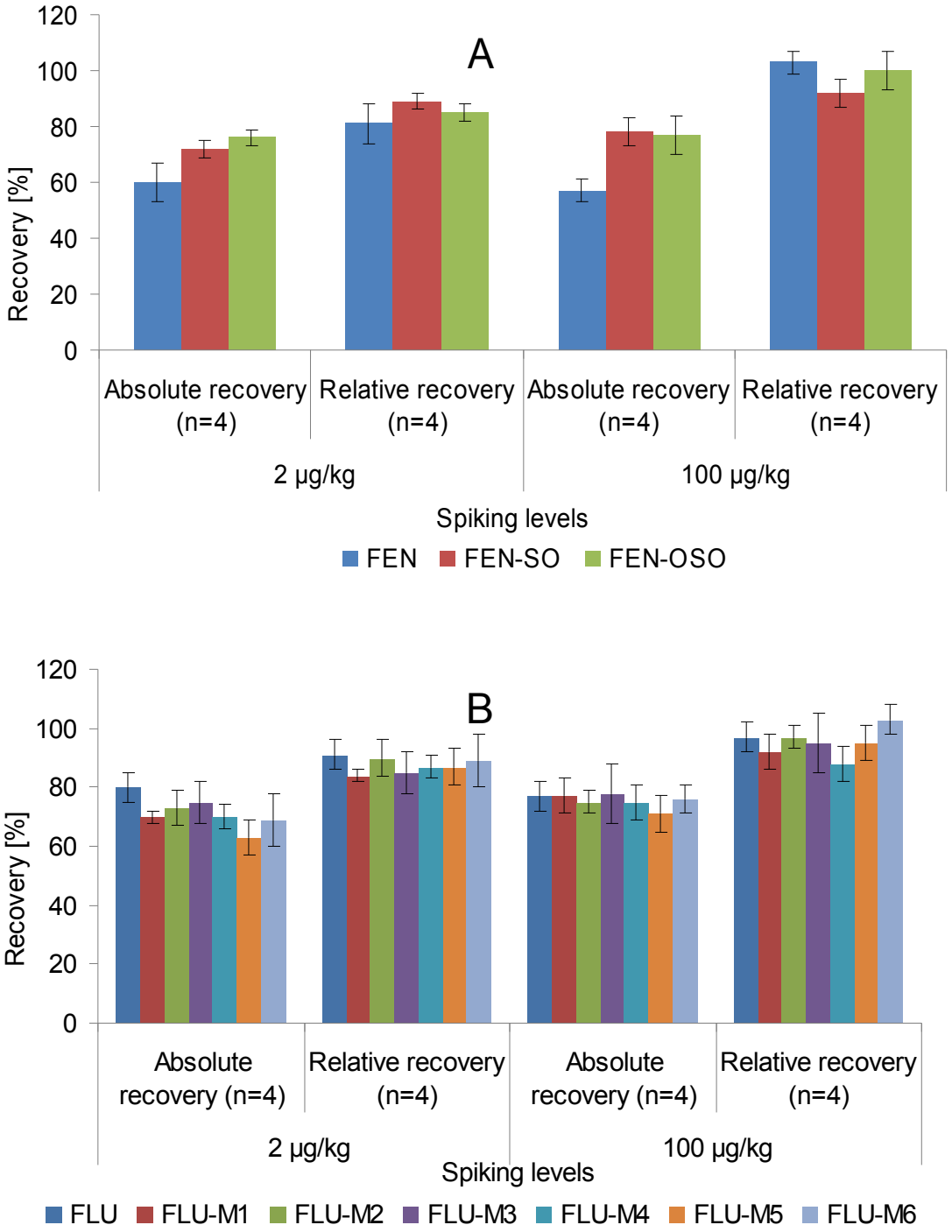


Figure 4.22 Recovery rates of A: fenbendazole and B: flubendazole with corresponding metabolites spiked in manured clay soil samples at different spiking levels and extracted with methanol/ethyl acetate mixture (1:4, v/v) at alkaline pH,

4.1.3.2 Ultrasound-assisted solvent extraction

In accordance with the analysis of lyophilized manure samples, manured sand soil samples were spiked with target compounds at 100 µg/kg and extracted with 100 mL methanol/ethyl acetate mixture (1:4, v/v) at alkaline pH using USE technique for 30 min. The extracted samples were rinsed twice with 100 mL of the solvent mixture for 15 min. The obtained recovery rates ranged from 88 to 93% with RSD ≤ 7% for all of the target compounds. Later on, 3 rinsing steps, two with 70 mL of and one with 60 mL the solvent mixture followed. Additionally, two spiking levels were used to check for their effects on the recovery. For this purpose, manured sand soil samples were spiked with the target compounds at 2 and 10 µg/kg. All compounds were extracted with recovery rates ranged from 89 to 107 and 86 to 119% with RSD ≤ 10 and 16%, respectively. Results of all target compounds extracted from manured sand and clay soil samples are given in **Figure 4.23.**

In manured clay soil samples at 2 and 50 µg/kg spiking levels, the mean recovery rates for all target compounds were from 97 to 112 % and 88 to 98 % with RSD ≤ 17 % and 14 %, respectively **(Figure 4.24)**. These results indicated that this method was feasible for analysis of the target compounds in different soil matrices even at low concentration levels. This may be attributed to USE accelerating some steps during the extraction procedure, e.g., releasing the analytes out of the solid matrices due to efficient contact between the solids and the solvent (Babic et al., 1998; Zhou et al., 2009). The compared results of recoveries between direct solvent extraction method with mechanical shaking and the ultrasound-assisted extraction are listed in **Table 4.4.** These results showed that the recoveries of the target compounds obtained by ultrasound-assisted extraction were matching with those obtained by the direct solvent extraction method. Thus, USE is considered as an alternative method for benzimidazole analysis in solid samples. **Figure 4.25** shows the analytical scheme of the optimized methods for analysis of target compounds in soil and manured soil samples.

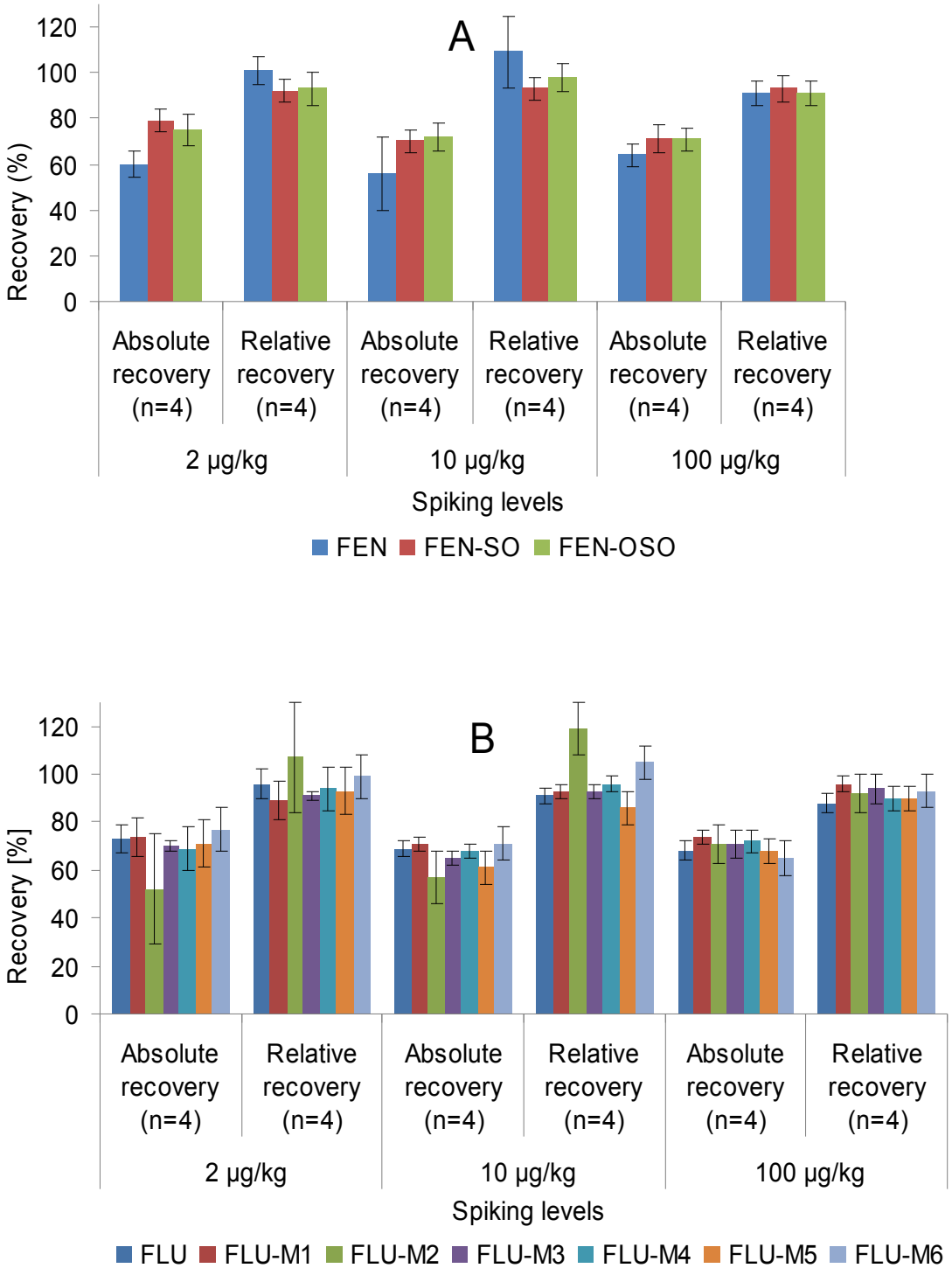


Figure 4.23: Recovery rates of A: fenbendazole and B: flubendazole with corresponding metabolites spiked in manured sand soil samples extracted by methanol/ethyl acetate mixture (1:4; v/v; pH 9.5) via ultrasound-assisted solvent extraction.

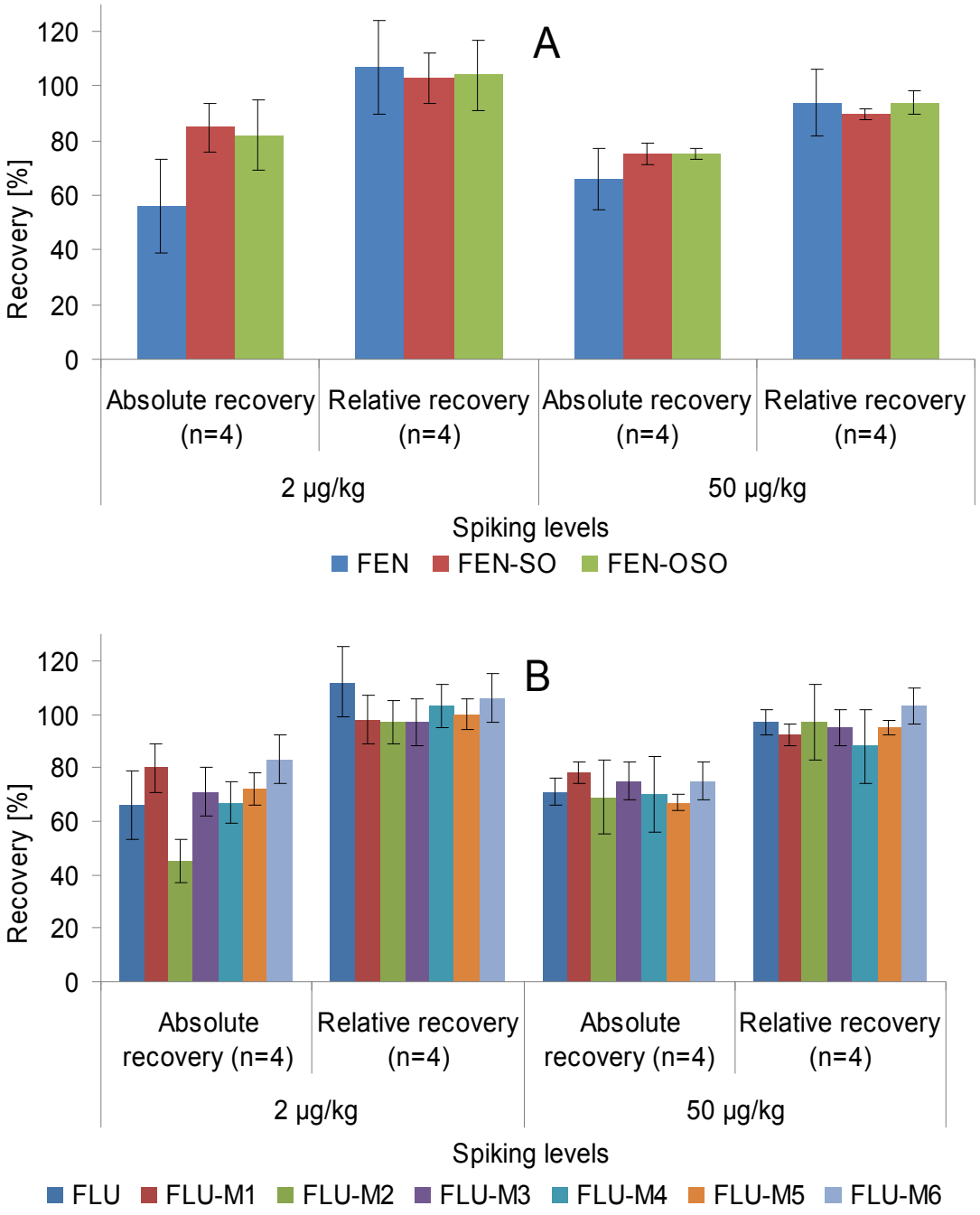


Figure 4.24: Recovery rates of A: fenbendazole and B: flubendazole with corresponding metabolites spiked in manured clay soil samples and extracted by methanol/ethyl acetate mixture (1:4; v/v; pH 9.5) via ultrasound-assisted solvent extraction.

Table 4.4: Recovery rates of fenbendazole and flubendazole with their corresponding metabolites in manured sand soil samples extracted by methanol/ethyl acetate mixture (1:4 v/v) at alkaline pH via ultrasound assisted solvent extraction and direct solvent extraction method.

Analytes	Manured sand soil samples			
	Ultrasound-assisted extraction		Direct solvent extraction	
	10 µg/kg		10 µg/kg	
	Absolute recovery (n=4)	Relative recovery (n=4)	Absolute recovery (n=4)	Relative recovery (n=4)
FEN	56 ± 16	109 ± 16	55 ± 8	94 ± 8
FEN-SO	70 ± 5	93 ± 5	73 ± 6	103 ± 6
FEN-OSO	72 ± 6	98 ± 6	72 ± 6	103 ± 6
FLU	69 ± 3	91 ± 3	70 ± 4	96 ± 4
FLU-M1	71 ± 3	93 ± 3	67 ± 4	99 ± 4
FLU-M2	57 ± 11	119 ± 11*	67 ± 5	98 ± 5
FLU-M3	65 ± 3	93 ± 3*	71 ± 8	94 ± 8
FLU-M4	68 ± 3	96 ± 3*	66 ± 4	93 ± 4
FLU-M5	61 ± 7	86 ± 7	62 ± 9	102 ± 9
FLU-M6	71 ± 7	105 ± 7	76 ± 16	110 ± 16

*n = 2

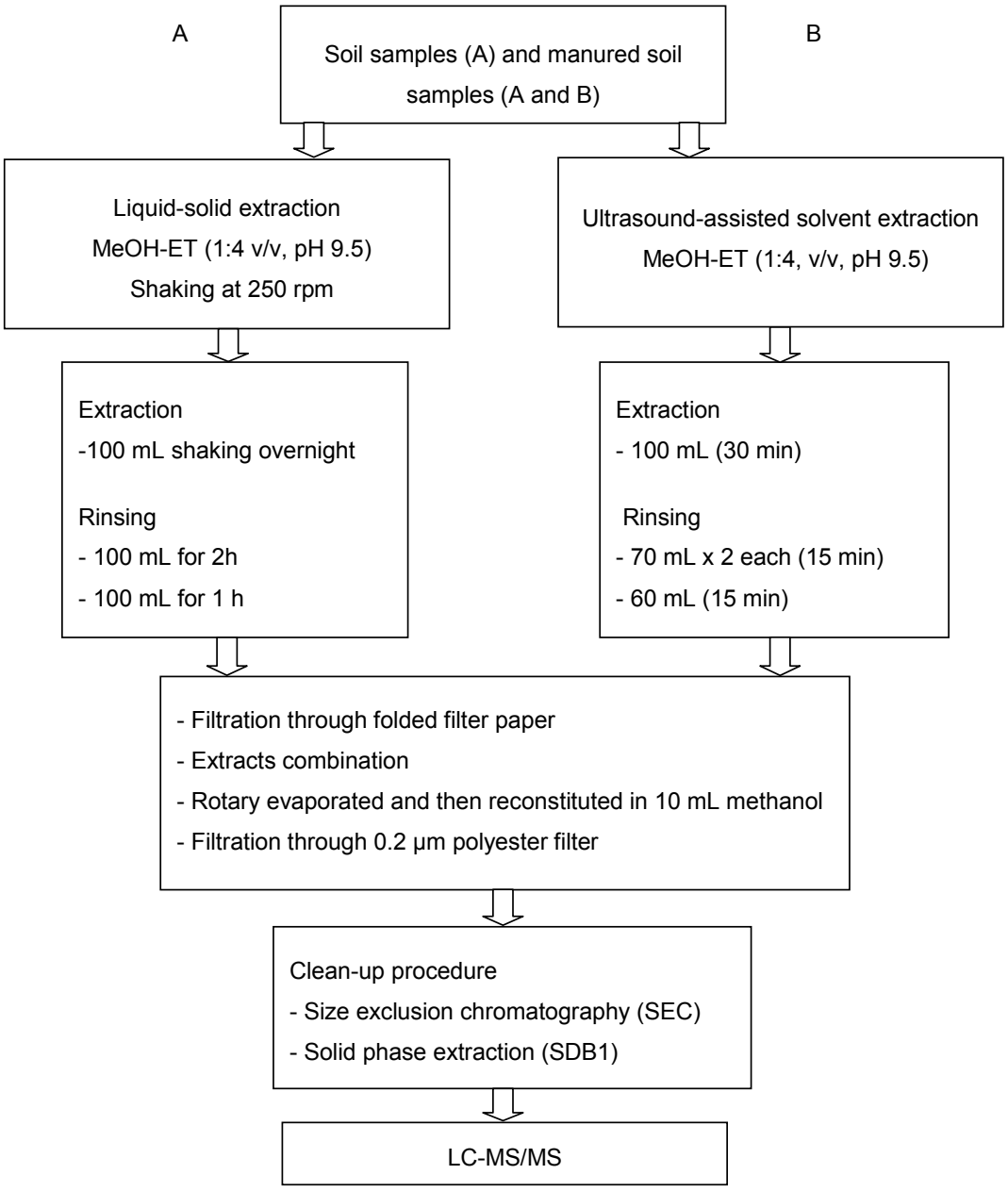


Figure 4.25: Analytical scheme of the optimized methods for target benzimidazoles analysis in soil and manured soil samples via different extraction methods.

4.2 Clean-up procedures

The clean-up process of the raw extracts is a further critical step with relevant impacts on the subsequent analytical procedure, especially, when LC/MS/MS operating in electrospray ionization is used for analysis of environmental samples of high complexity and variability. The matrix components in manure, soil and manured soil samples can produce undesirable

and unpredicted effects on the responses of target compounds. Therefore, sophisticated clean-up procedures were needed to meet the requirements of the analytical quality assurance (**Figure 4.26**).

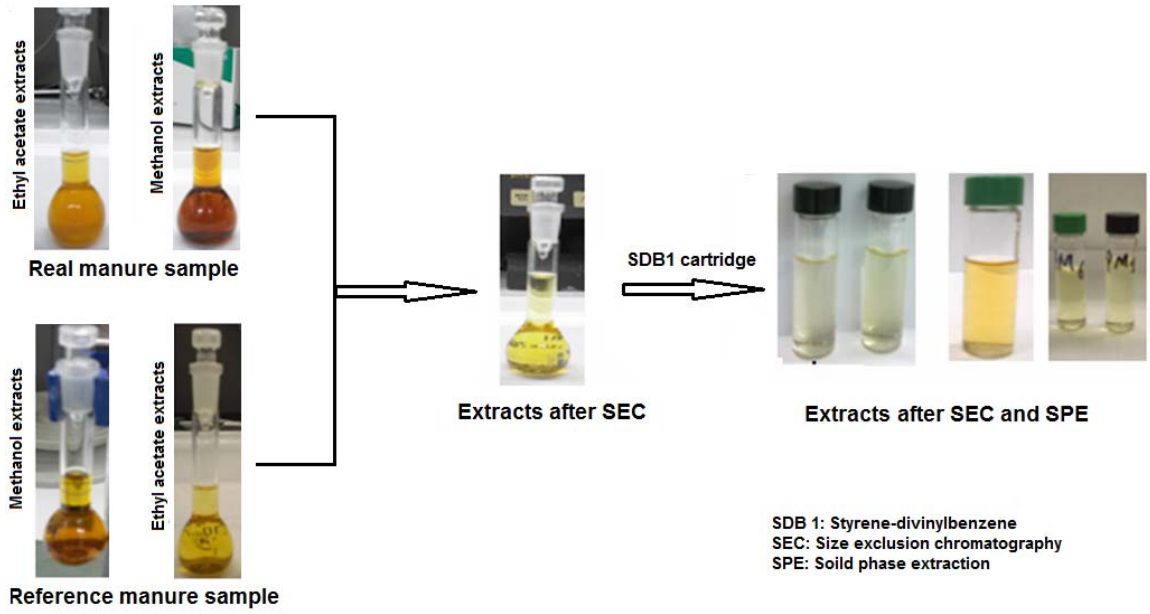


Figure 4.26: Effect of clean-up procedure on the extracts of real and reference manure samples extracted by ethyl acetate or methanol.

4.2.1 Size exclusion chromatography

The raw extracts produced by different extraction procedures were initially filtered via 2-µm syringe polyester filters. Subsequently, SEC clean-up step was conducted using a Sephadex column which was successfully used by Kreuzig et al. (2007) to clean-up the extracts of manure and soil samples containing FEN and FLU extracted by direct solvent extraction using ethyl acetate. In this method, methanol containing 0.01 M acetic acid as eluent with a flow rate of 5 mL/min was applied as the optimum conditions for this clean-up process. At the beginning of presented method development, the same stationary and mobile phase was also used. Considering the widely different physico-chemical properties of the target compounds under study, the method was modified using methanol instead of acidified methanol to decrease the losses up to 19 % especially for the more polar metabolites. Due to the high complexity and variability of the analyzed matrices, extracts of different quality were obtained. Therefore, it was necessary to add another clean-up step to improve the quality of the final extracts.

4.2.2 Solid phase extraction

Oasis HLB and SDB1 were successfully used to extract these compounds from surface water with recovery rates > 84 % for all of the target compounds. Concentrated extracts were diluted with 200 mL of deionized water, acidified using formic acid to pH 2.3 and then loaded on the conditioned cartridges. Both cartridges were processed individually and it was found that the first set of Oasis HLB cartridges were totally blocked after passing through of only 90 mL of the samples. During extraction procedure, it was observed that the particles in diluted manure matrix due to emulsion formation quickly slowed down the flow, and finally blocked the Oasis HLB cartridges. Because of Oasis HLB could not be used under these conditions, it was excluded from the clean-up procedure. To avoid this problem with SDB1, a pre-filtration with 0.6-µm glass fiber filters pre and post washed with deionized water was carried out before extraction using SDB1. However, this filtration step did not efficiently remove these particles. Thus, 5 g of sodium chloride (NaCl) were added to the diluted extracts (200 mL), where the emulsion was completely destroyed as it can be seen in **Figure 4.27**.

For elution of the loaded target compounds from these polymeric cartridges, solvents of different polarities and desorption power, e.g., methanol, acetonitrile and ethyl acetate were tested. The obtained recoveries were compared with the recoveries obtained by SDB1 cartridges from surface water at pH 2.3 using methanol as eluting solvent, where all target compounds were efficiently extracted with recovery rates ranging from 84 to 116 % with RSD < 11 % and successfully eluted by 2 x 5 mL methanol. Under the same extraction conditions, recoveries rates of all target compounds were decreased in case of diluted manure samples and ranged from 56 to 71 % with RSD < 16 % when 2 x 5 mL methanol were used for elution of the target compounds (**Figure 4.28**).

Figure 4.27: Effect of sodium chloride addition on the formation of emulsion during filtration by glass fiber filters.

The obtained recovery rates of FLU-M1, FLU-M2, FLU-M3 and FLU-M4 were 5 to 20 % when acetonitrile and ethyl acetate were used to elute these analytes, while the average recovery of FEN and FEN-SO was < 35 %, when acetonitrile was used. This decrease of the obtained recoveries, in the case of diluted manure matrix extracted under the same conditions including pH, SDB1 and methanol as desorption solvent, may be attributed to competition of dissolved organic matter (DOM) and other compounds present in diluted manure matrix with target compounds for the active adsorbing sites. The same situation was also observed for diluted extracts of soil and manured soil. For this reason the absolute and relative recoveries were calculated to identify the extraction efficiencies (relative recoveries) as well as to test the losses during clean-up procedures. To study the loss of target compounds during the clean-up procedure (SEC and SPE), the tested concentration was spiked at three different steps of the procedure. First (A), the tested concentration was spiked into the sample prior to extraction. Second (B), it was spiked immediately at the beginning of the clean-up procedure (SEC and SPE). Finally (C), this concentration was spiked after SEC and SPE (C) into the 5 mL methanolic extract. The recoveries were calculated as shown in Eq. 4.1 to 4.3.

$$\text{The absolute recovery} = A/C \qquad \text{(Eq. 4.1)}$$

$$\text{Relative recovery (extraction efficiency)} = A/B \qquad \text{(Eq. 4.2)}$$

$$\text{The losses during the clean up procedures} = B/C \qquad \text{(Eq. 4.2)}$$

Using the latter equation absolute recoveries of the target compounds obtained after the clean-up procedure including SEC and SDB1 were calculated using different matrices, such as manure, soil and manured soil using different extraction procedures at different spiking levels as it can be seen in **Tables 4.5** to **4.8.**

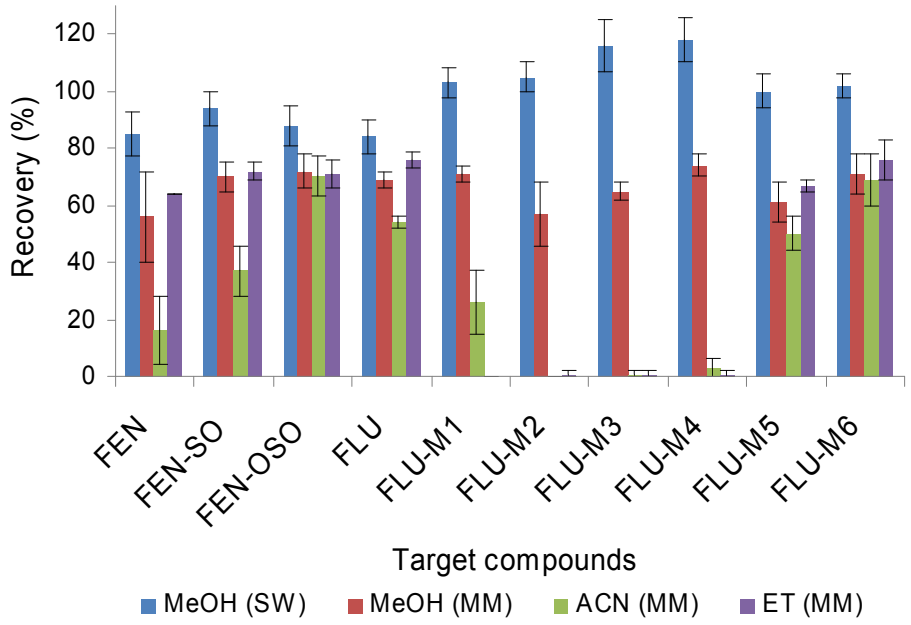


Figure 4.28: The obtained recoveries of the target compounds extracted from diluted manure matrix (MM) in deionized water using SDB1 at pH 2.3 and different eluting solvents including methanol, acetonitrile and ethyl acetate, compared with recoveries of the target compounds extracted from surface water (SW) using methanol as eluent.

4.2.3 Lipid removal using n-hexane

It should be noted that the losses were not only related to SPE process but also some losses were observed during filtration of diluted extract using 0.6-µm glass fiber filters. Approximately 25 % of FEN was lost while the losses of the other target compounds were less than 7%. It can be assumed that more hydrophobic FEN is more lipid soluble and lost during glass fiber filtration. This is also the same reason for the higher losses of FEN and FLU when lyophilized manure sample were washed with 100 mL n-hexane to remove lipids as a preliminary clean-up step. The losses were 31 % for FEN and 57 % for FLU, while the losses were < 14 % for the other target compounds. Moreover, losses ranged from 5-19 % were observed when a small volume of n-hexane was used (10 mL) as washing step for SDB1 cartridges after sample loading (**Tables 4.9).** Therefore, the use of n-hexane was excluded from this clean-up procedure.

Table 4.5: The mean absolute recovery rates of the clean-up procedure including SEC and SDB1 for the fenbendazole and flubendazole with corresponding metabolites spiked at different concentrations into manure extracts produced by different extraction methods (n=2).

Analytes	Direct ethyl acetate extraction from liquid manure			Direct methanol extraction After lyophilization			Extraction using MeOH/ET (1:4, v/v) After lyophilization		
	2 µg/kg	50 µg/kg	100 µg/kg	4 µg/kg	50 µg/kg	100 µg/kg	4 µg/kg	50 µg/kg	100 µg/kg
FEN	75 ± 11	55 ± 12	74 ± 12	44 ± 8	54 ± 2	60 ± 7	47 ± 11	51 ± 1	57 ± 5
FEN-SO	78 ± 13	73 ± 12	77 ± 3	65 ± 5	86 ± 4	94 ± 4	80 ± 3	74 ± 5	74 ± 9
FEN-OSO	79 ± 2	74 ± 12	75 ± 2	63 ± 5	80 ± 5	86 ± 4	73 ± 3	76 ± 6	73 ± 8
FLU	69 ± 4	74 ± 10	80 ± 4	63 ± 7	77 ± 5	82 ± 5	67 ± 1	72 ± 1	79 ± 7
FLU-M1	79 ± 4	75 ± 11	76 ± 4	67 ± 6	85 ± 6	89 ± 3	77 ± 3	69 ± 24	90 ± 10
FLU-M2	60 ± 4	39 ± 20	72 ± 4	63 ± 6	74 ± 5	84 ± 3	72 ± 3	75 ± 4	74 ± 8
FLU-M3	56 ± 5	56 ± 19	74 ± 1	68 ± 5	78 ± 7	71 ± 5	84 ± 1	77 ± 5	84 ± 2
FLU-M4	66 ± 5	39 ± 16	76 ± 5	68 ± 5	85 ± 4	93 ± 4	71 ± 3	75 ± 1	85 ± 3
FLU-M5	79 ± 3	71 ± 16	76 ± 3	69 ± 5	81 ± 5	89 ± 2	77 ± 3	75 ± 5	81 ± 9
FLU-M6	71 ± 5	72 ± 10	77 ± 5	85 ± 11	79 ± 6	82 ± 8	67 ± 3	85 ± 8	79 ± 11

Table 4.6: The mean absolute recovery rates of the clean-up procedure including SEC and SDB1 for the fenbendazole and flubendazole with corresponding metabolites spiked at different concentrations into soil extracts produced by direct solvent extraction using methanol/ethyl acetate (1:4, v/v) at alkaline pH (n=2).

Analytes	Sand soil				Clay soil		
	2 µg/kg	4 µg/kg	40 µg/kg	100 µg/kg	4 µg/kg	40 µg/kg	100 µg/kg
FEN	49 ± 10	69 ± 0	70 ± 7	62 ± 2	67 ± 7	66 ± 3	72 ± 8
FEN-SO	91 ± 14	86 ± 4	82 ± 5	92 ± 4	80 ± 2	78 ± 3	85 ± 4
FEN-OSO	85 ± 10	80 ± 3	83 ± 3	84 ± 2	77 ± 2	78 ± 3	83 ± 3
FLU	82 ± 8	81 ± 4	80 ± 1	82 ± 3	77 ± 2	74 ± 1	80 ± 5
FLU-M1	85 ± 9	78 ± 5	90 ± 3	86 ± 5	81 ± 1	81 ± 1	86 ± 3
FLU-M2	87 ± 12	81 ± 1	79 ± 1	75 ± 21	81 ± 4	84 ± 2	83 ± 8
FLU-M3	87 ± 11	76 ± 2	88 ± 2	82 ± 9	80 ± 1	80 ± 1	86 ± 3
FLU-M4	74 ± 10	55 ± 25	90 ± 4	55 ± 15	73 ± 3	82 ± 1	70 ± 8
FLU-M5	83 ± 3	67 ± 3	79 ± 1	72 ± 1	76 ± 4	79 ± 3	76 ± 13
FLU-M6	86 ± 13	81 ± 5	84 ± 2	83 ± 3	77 ± 4	75 ± 3	85 ± 6

Table 4.7: The mean absolute recovery rates of the clean-up procedure including SEC and SDB1 for the fenbendazole and flubendazole with corresponding metabolites spiked at different concentrations into manured soil extracts produced by direct solvent extraction using methanol/ethyl acetate (1:4, v/v) at alkaline pH (n=2).

Analytes	Manured sand soil			Manured clay soil	
	2 µg/kg	10 µg/kg	50 µg/kg	2 µg/kg	100 µg/kg
FEN	54 ± 3	59 ± 5	52 ±1	72 ± 9	55 ± 4
FEN-SO	75 ± 9	71 ± 8	73 ± 6	82 ± 2	85 ± 5
FEN-OSO	69 ± 3	70 ± 9	69 ± 2	90 ± 3	77 ± 4
FLU	70 ± 11	71 ± 5	70 ± 5	88 ± 2	79 ± 1
FLU-M1	68 ± 5	68 ± 1	67 ± 2	83 ± 11	84 ± 3
FLU-M2	67 ± 4	68 ± 2	63 ± 7	82 ± 1	78 ± 2
FLU-M3	74 ± 3	75 ± 3	70 ± 4	87 ± 2	82 ± 3
FLU-M4	75 ± 5	70 ± 1	69 ± 9	80 ± 1	85 ± 1
FLU-M5	69 ± 4	62 ± 5	54 ± 5	73 ± 1	74 ± 1
FLU-M6	65 ± 20	67 ± 8	68 ± 1	77 ± 15	73 ± 9

Table 4.8: The mean absolute recovery rates of the clean-up procedure including SEC and SDB1 for fenbendazole and flubendazole with corresponding metabolites spiked at different concentration levels into manured soil extracts produced by ultrasound-assisted extraction using methanol/ethyl acetate mixture (1:4, v/v) at alkaline pH.

Analytes	Manured sand soil			Manured clay soil	
	2 µg/kg	10 µg/kg	100 µg/kg	2 µg/kg	50 µg/kg
FEN	60 ± 2	52 ± 5	71 ± 1	53 ± 3	65 ± 1
FEN-SO	85 ± 2	75 ± 7	77 ± 2	83 ± 6	80 ± 3
FEN-OSO	80 ± 4	70 ± 5	79 ± 2	79 ± 5	81 ± 1
FLU	78 ± 1	76 ± 3	76 ± 2	59 ± 4	79 ± 0
FLU-M1	84 ± 4	76 ± 4	84 ± 1	81 ± 3	84 ± 2
FLU-M2	49 ± 28	48 ± 5	74 ± 5	47 ± 23	74 ± 1
FLU-M3	76 ± 6	70 ± 9	78 ± 6	76 ± 13	77 ± 4
FLU-M4	74 ± 6	69 ± 3	76 ± 8	65 ± 15	75 ± 5
FLU-M5	76 ± 3	70 ± 6	75 ± 1	72 ± 5	71 ± 0
FLU-M6	78 ± 7	68 ± 11	71 ± 6	78 ± 10	85 ± 4

Table 4.9: Losses of the target compounds during the steps of clean-up optimization.

Analytes	Losses during clean-up [%]		
	A	B	C
FEN	57	25	17
FEN-SO	11	0	0
FEN-OSO	11	5	1
FLU	31	7	2
FLU-M1	12	0	0
FLU-M2	13	0	1
FLU-M3	14	0	0
FLU-M4	11	0	0
FLU-M5	10	3	0
FLU-M6	10	5	0

A: Rinsing step with 100 mL n-hexane to the lyophilized samples extracted with methanol (Excluded).

B: Glass fibre filtration step (included in the optimized procedure).

C: SDB1 washing step after loading the samples with 10 mL n-hexane (Excluded).

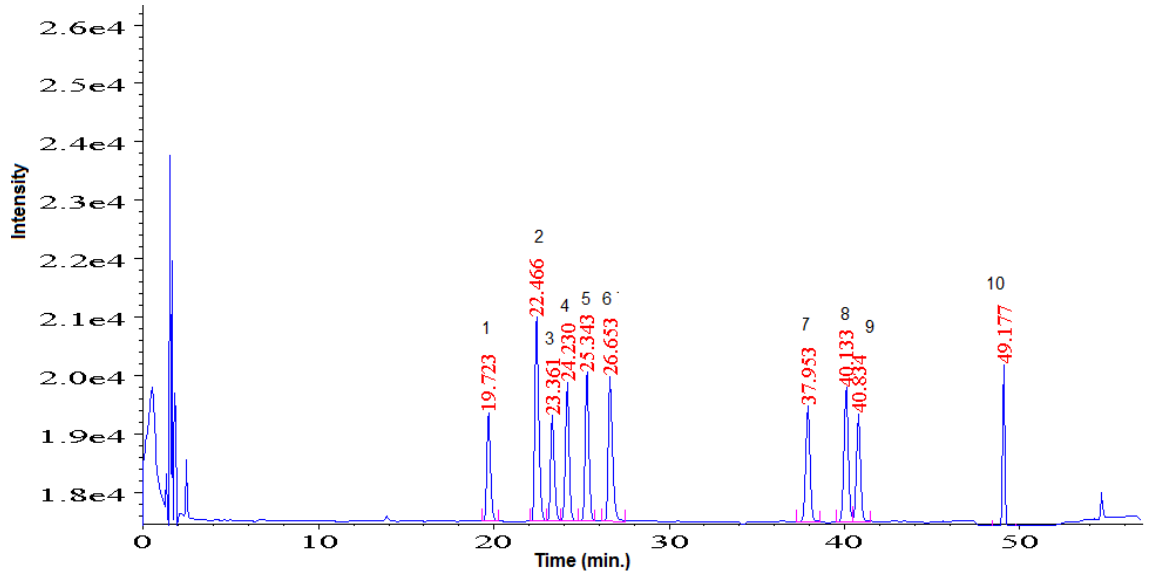
The results of the absolute recoveries of the clean-up procedure demonstrated that the relevant effects of the sample heterogeneity during the clean-up procedure, especially at SPE step should be considered. The mean losses of the target compounds were 10 to 40% except FEN where the losses may be increased to > 50 % due to glass fibre filtration step, especially at spiking levels close to the limit of quantitation.

4.3 Optimization of HPLC/UVD analysis

Several parameters, such as peak shape, resolution and run time, were considered in the chromatographic method developed for simultaneous analysis of FEN and FLU with corresponding metabolites. Therefore, the selection of stationary and mobile phases in addition to the gradient programming, were the main relevant parameters. Benzimidazoles are intermediately polar and weakly basic compounds with wide ranges of physio-chemical properties. Hence, reversed phase (RP) including C8 or C18 columns are frequently used in the analysis of these compounds (Danaher et al., 2007). Most of these stationary phases are silica-based bonded phases chemically modified at their surfaces in order to reduce the polarity. The basic compounds such as benzimidazole compounds under acidic condition are

usually suffering from peak tailing due to strong silanophilic interaction between protonated analytes and these stationary phases containing free silanol groups. The majority of the HPLC methods for the determination of benzimidazoles have been thus developed using ion pair chromatography to solve this problem (Botsoglou et al., 1997). In this work, however, Zorbax Eclipse XDB endcapped columns (150 mm x 4.6 mm, 5 µm), which are specially designed to reduce or eliminate this strong adsorption of basic drugs, were selected. Eclipse XDB columns realize a wide useable range of pH from 2 to 9 (Agilent ZORBAX Column Selection Guide for HPLC, Internet). Thus, it was well suited for the initial method development at pH 3 to provide high resolution and excellent peak shape of target compounds without any needs for ion pair reagents. For separation a complex sample mixture such as FEN and FLU with corresponding metabolites, a gradient HPLC system instead of an isocratic system was used. In the terms of physio-chemical properties, considerable differences in the chromatographic behavior between parent compounds and their corresponding metabolites were expected. Therefore, the more polar FLU metabolites and FEN-OSO characterized were by rapid elution times, necessitating the use of a high initial aqueous percentage in the mobile phase to retain these metabolites. Thereafter, the acetonitrile contents increased over time until the most hydrophobic analytes were eluted. Moreover, the acetonitrile content increased during the analysis to 100% after elution of highly hydrophobic substances for few minutes to clean the column. Finally, the initial percentage of the mobile phase was adjusted again during the equilibration period.

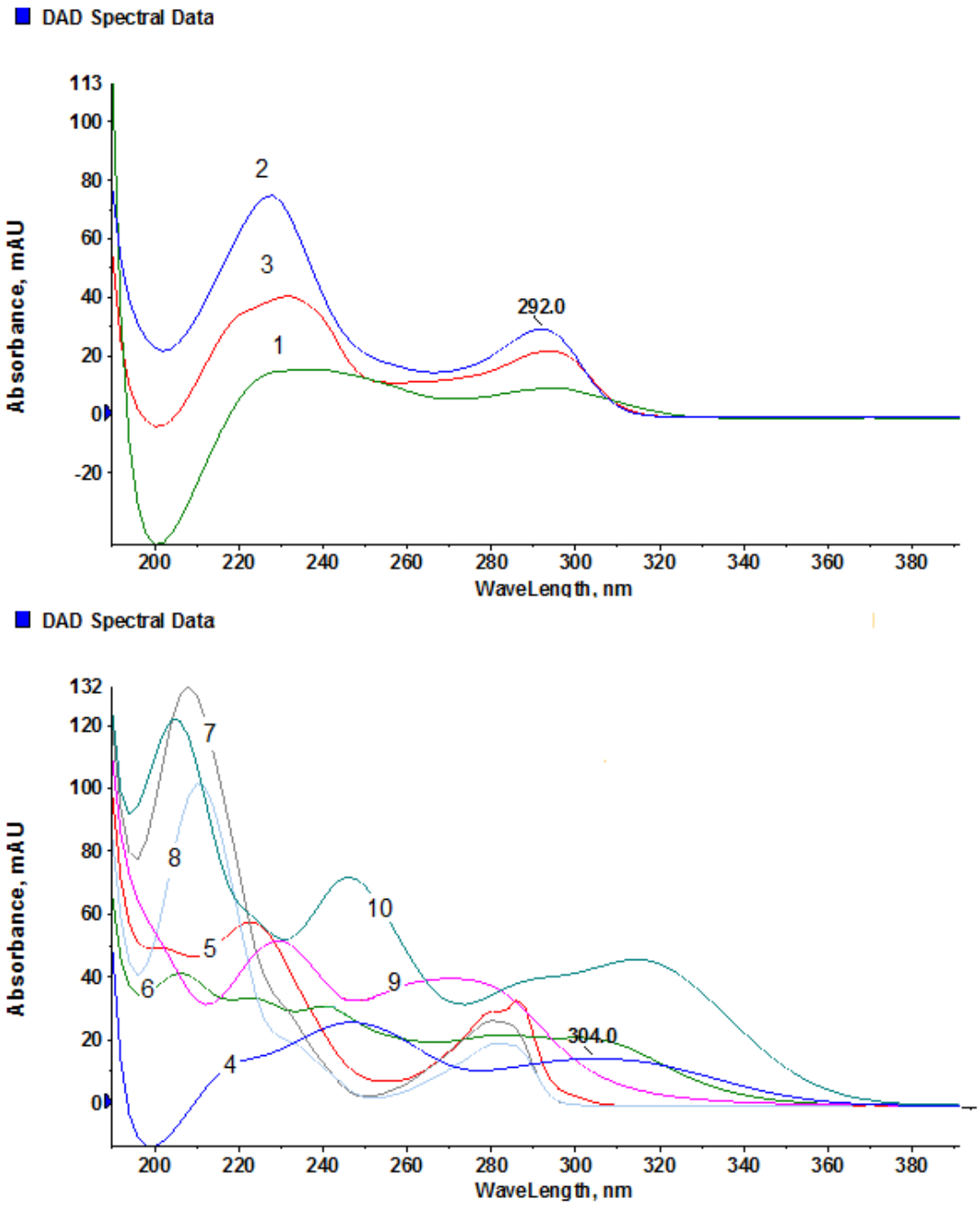
In addition to their stability over a wide pH range and at high temperature ≤ 90 ºC, endcapped columns are able to prevent the collapse of stationary phase due to high percentage of the aqueous phase at the beginning of the gradient system (Díaz-Cruz and Barceló, 2006). For this purposes, Zorbax endcapped C18 columns are equipped with densely covered and strictly protected dimethyl-n-octadecylsilane as present in Eclipse XDB-C18 columns, respectively. A number of preliminary experiments were performed to determine the optimum conditions for target compounds separation using these columns. All of the target compounds were separated using a mobile phase consisted of 0.5 % formic acid in water as mobile phase A. Phase B consisted of acetonitrile and phase A (75:25, v/v), respectively, using the Zorbax Eclipse XDB-C18 column (150 mm x 4.6 mm, 5 µm; Agilent Technologies, Santa Clara, CA, USA). The injection volume was 10 µL. Flow rate was 1 mL/min at ambient temperature (**Figure 4.29**). Using the mentioned chromatographic conditions, well resolved sharp peaks were achieved without any peak tailing for all target compounds. It should be noted that addition of formic acid was important for analytes separation in order to decrease the number of free silanol groups, improving the peak shape (Maraschiello et al., 2001).



1: FLU-M3, 2: FLU-M5, 3: FLU-M4, 4: FEN-SO, 5: FLU-M1, 6: FLU-M2,
7: FEN-OSO, 8: FLU, 9: FLU-M3, 10: FEN

Figure 4.29: HPLC/UVD chromatogram of fenbendazole and flubendazole with corresponding metabolites at 10 ng/µL methanol, recorded at λ = 280nm.

At the beginning of HPLC/UVD experiments, wavelength selection was based on the already published values (Danaher et al., (2007). As applied by constant wavelength detectors, λ = 254 nm was used first for the target compounds detection. However, sufficient signal intensity for some of these compounds was not found because the detection did not meet the substance specific UV absorption maxima. The problem overcame by coupling of HPLC with the photodiode array detector (HPLC/DAD). According to the recorded UV absorption spectra, the maximum absorbance of the target benzimidazoles was identified at λ = 280 nm (**Figure 4.30**) Hence, substance identification and quantitation based on retention time comparison and specific wavelength detection.



1: FEN, 2: FEN-SO, 3: FEN-OSO, 4: FLU, 5: FLU-M1, 6: FLU-M2,
7: FLU-M3, 8: FLU-M4, 9: FLU-M5, 10: FLU-M6

Figure 4.30: UV absorption spectra of fenbendazole and flubendazole with corresponding metabolites at 25 ng/µL methanol, recorded by HPLC/DAD.

4.4 Optimization of LC/MS/MS experiments

The mass spectrometric behavior of the parent compounds using the methanolic standard solutions was initially examined by direct infusion analysis using Q1 and PI scans. For this purpose, different ionization sources, e g., ESI and APCI, as well as different ionisation modes, e g., positive and negative ionization modes were applied. All the target compounds were more sensitively detectable with ESI in positive ionization mode. Merely, signals of low intensities were recorded for all of the compounds in negative ionization mode. During the acquisition of the full scan spectra of benzimidazole compounds, the precursor ions were determined for all analytes in form of $[M+H]^+$. Abundant $[M+Na]^+$ sodium adducts were observed for FEN, FEN-SO, FEN-OSO and FLU. Therefore, addition of formic acid was necessary to avoid adduct formation. Without any additives, alkali metal content of the samples or the mobile phase can strongly form adducts and affecting the final method sensitivity. It should be noted that sodium adducts of FLU and FLU-M1 were also reported in the study performed by Nobilis et al. (2007). Moreover, formic acid is well known as efficient reagent used to prevent adduct formation and to promote the formation of the precursor ions $[M+H]^+$ in positive ESI mode via enhancement of the protonation process as described by Díaz-Cruz and Barceló et al. (2006). **Figure 4.31** shows $[M+H]^+$ for FLU compound with $[M+Na]^+$, where the adduct ion formation was avoided after formic acid addition. Further mass spectra are shown in the **Appendix, Figure A1**.

Infusion analysis of individual compounds also permitted the selection of product ions and the optimization of compound dependent parameters to enhance the method sensitivity for each compound. **Figure 4.32** to **4.35** show Q1 and PI spectra of FLU, FLU-M1, FEN and FEN-SO. Further mass spectra in positive and negative ionization modes are presented in the **Appendix, Figure A2 and A3**. During this procedure, it was necessary to select different product ions for each target compounds to have high degree of method specificity, especially in one mixture containing isobaric molecules such as FEN-SO and FLU-M1 (m/z 315, **Figure 4.33** and **4.35**) or six metabolites of one parent compound with nearly the same fragmentation patterns, e.g., FLU and FLU-M2 as shown in **Figure 4.36**. In this method, the transition with highest intensity was selected as quantitation ion and another one as qualification ion considering the above mentioned concept about specificity. For this purpose, full scan spectra, e.g., Q1 and product ion of the target compounds were determined using methanol acidified with formic as LC-eluent.

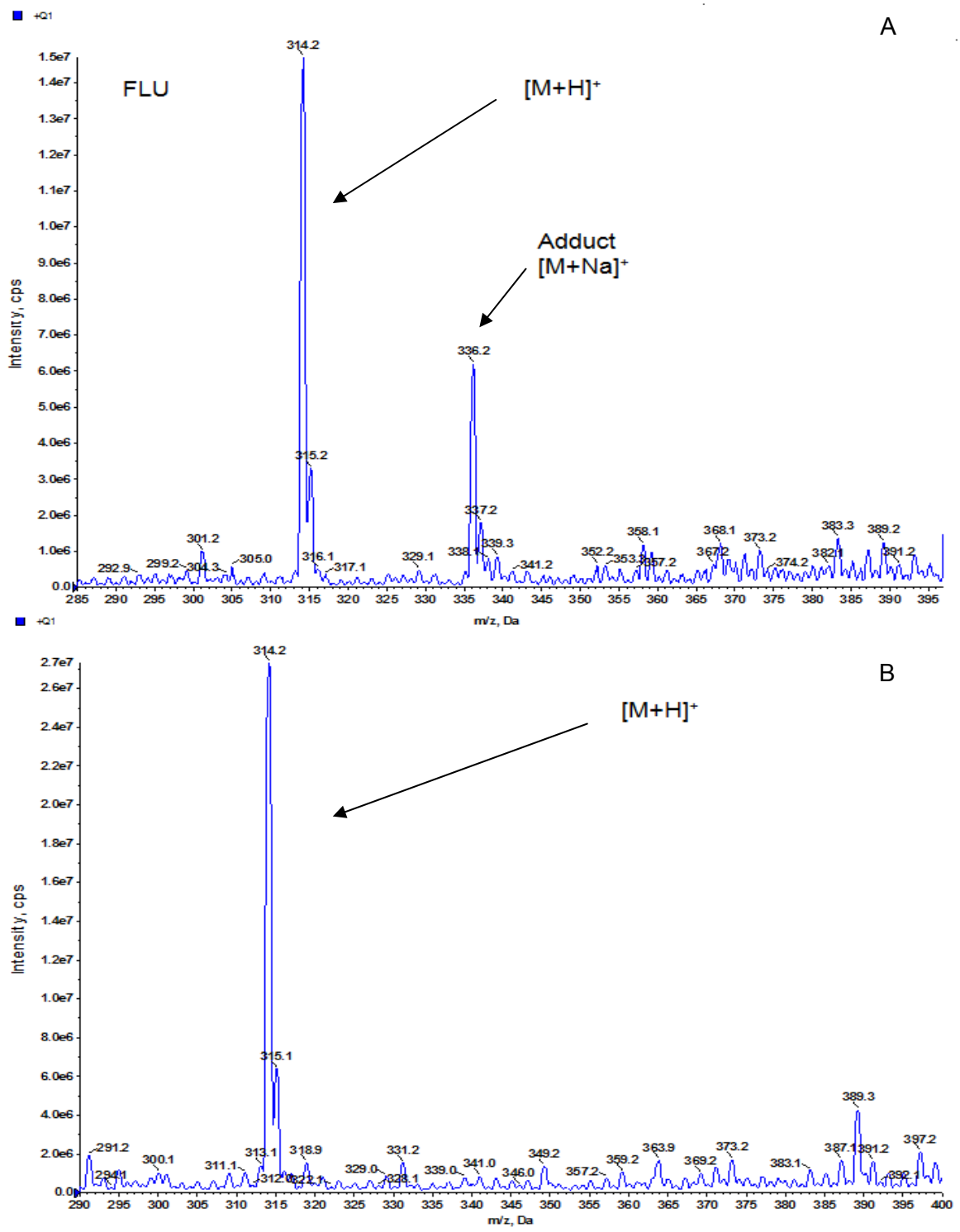


Figure 4.31: Mass spectra of flubendazole recorded in the full scan mode (ESI+, Q1 scan) showing A: the precursor ion with the corresponding sodium adduct in methanol, B: sodium adduct was avoided by adding formic acid in methanol.

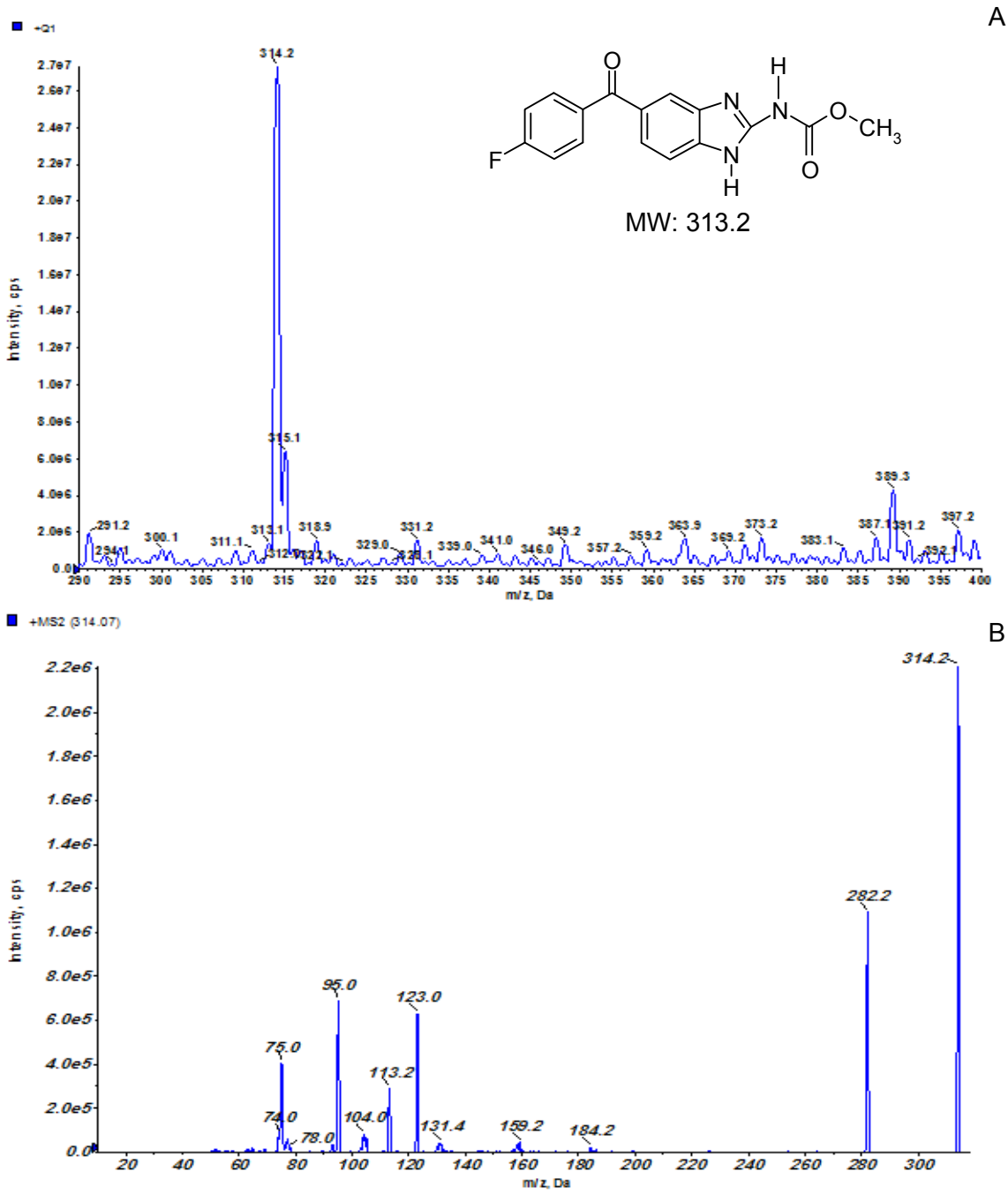


Figure 4.32: Mass spectra of flubendazole A: Q1 (MS) showing precursor ion of 314.2 m/z, B: product ion (MS/MS) showing different fragments of flubendazole (50 pg/µL methanol) in positive electrospray ionization mode (ESI+).

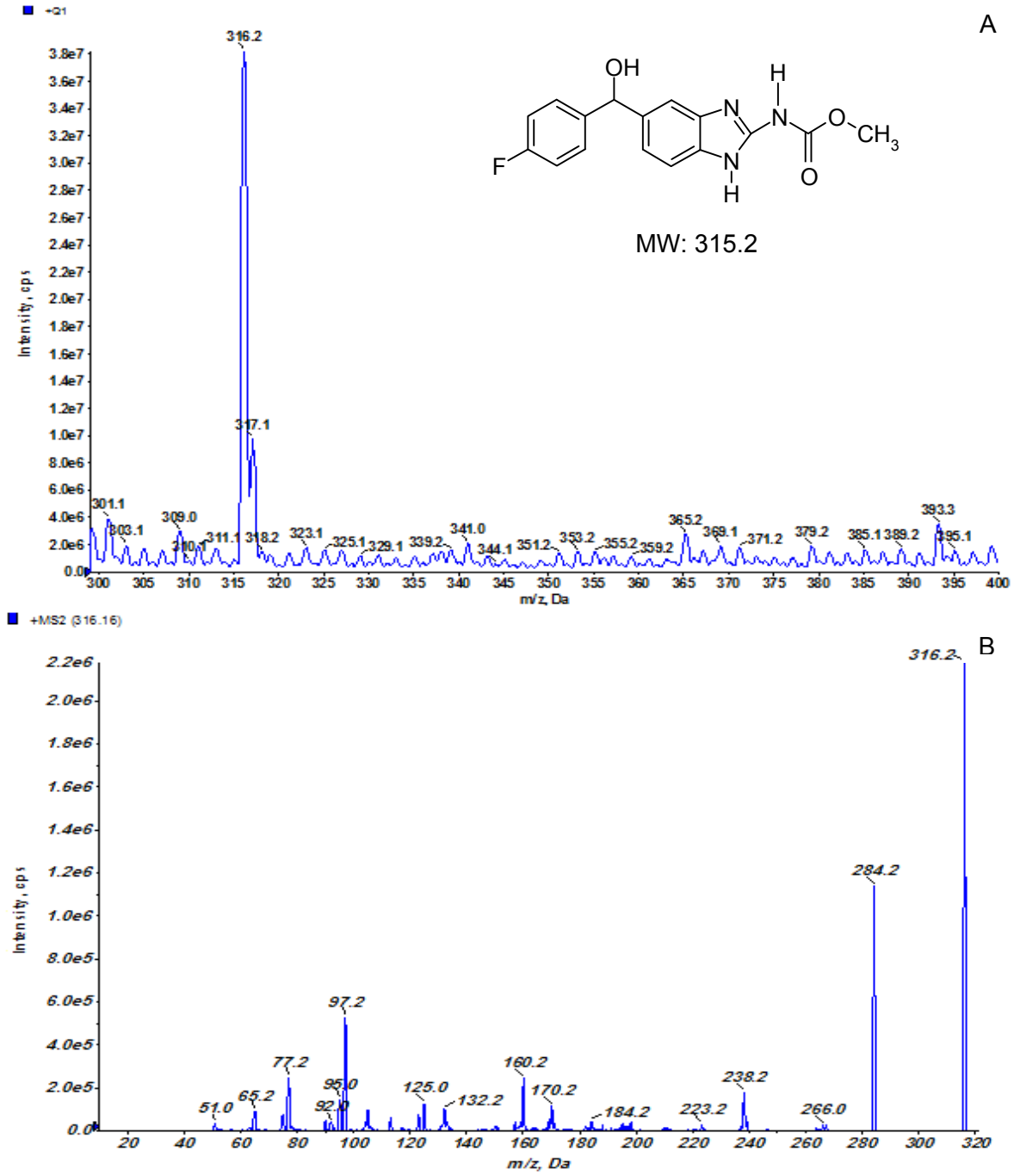


Figure 4.33: Mass spectra of flubendazole metabolite (FLU-M1) A: Q1 (MS) showing the precursor ion of 316.2 m/z, B: product ion (MS/MS) showing different fragments (50 pg/µL methanol) in positive electrospray mode.

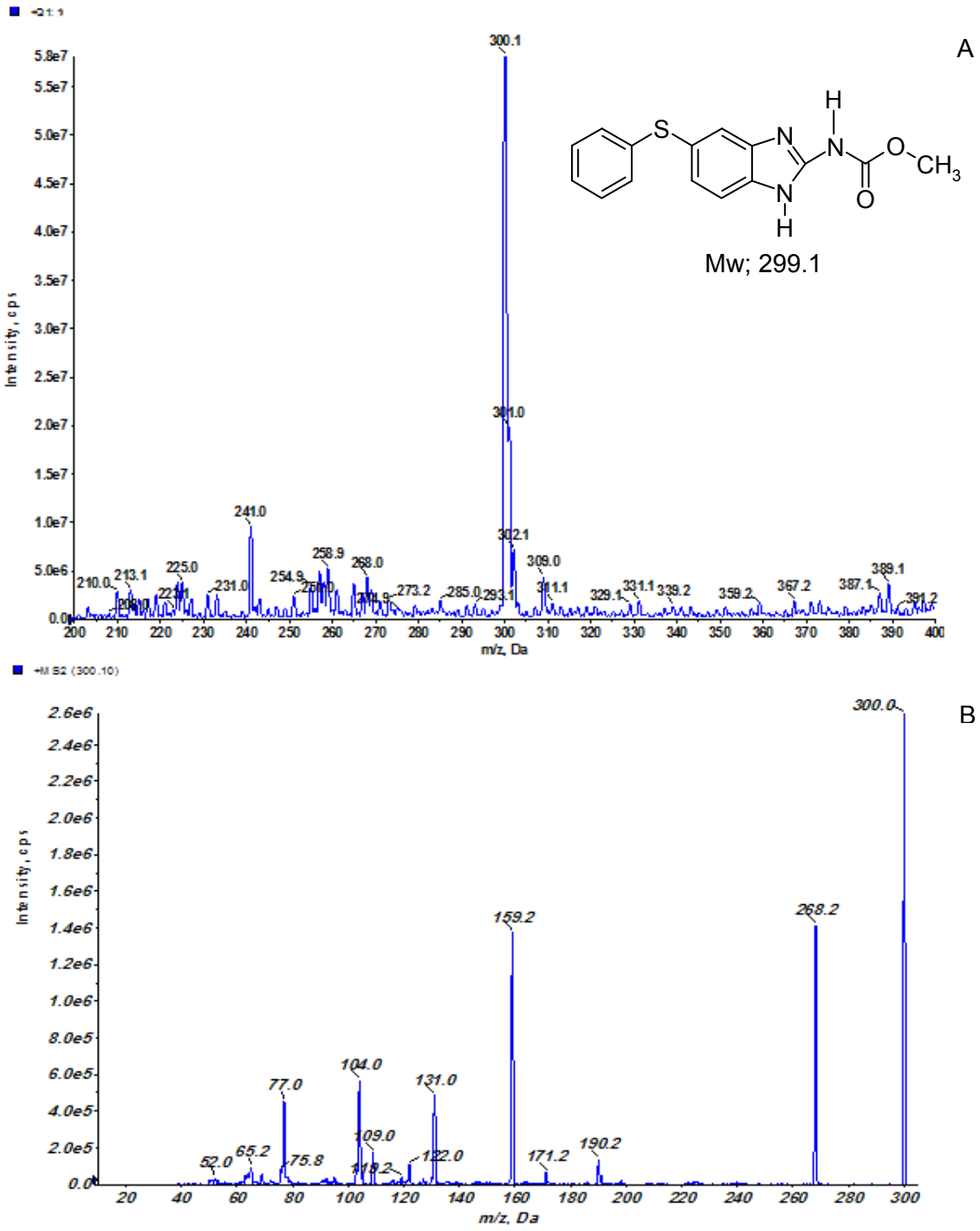


Figure 4.34: Mass spectra of fenbendazole A: Q1 (MS) showing the precursor ion of 300 m/z, B: product ion (MS/MS) showing different fragments of fenbendazole (50 pg/µL methanol) in positive electrospray mode.

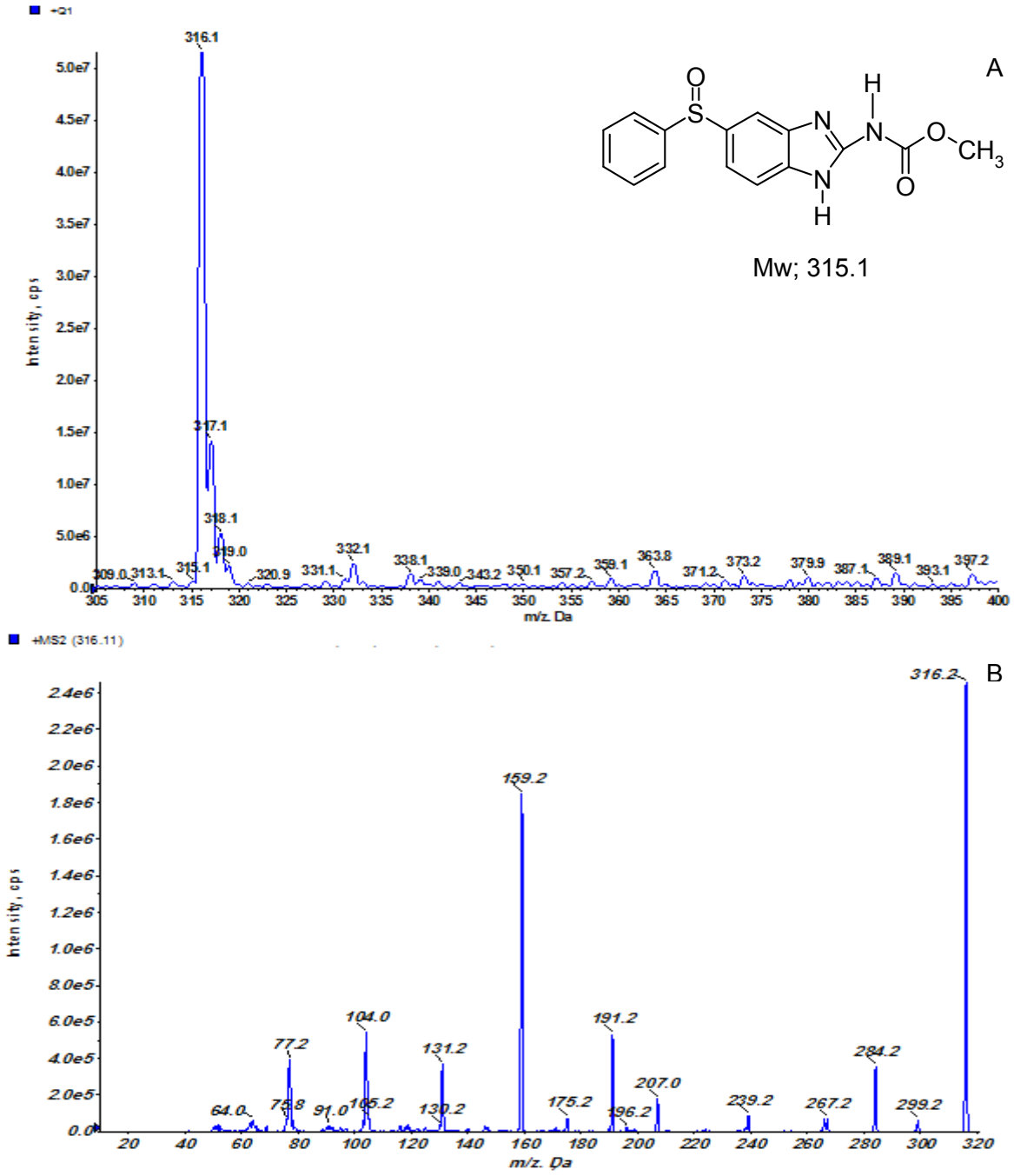


Figure 4.35: Mass spectra of fenbendazole sulfoxide A: Q1 (MS) showing the precursor ion of 316.2 m/z, B: product ion (MS/MS) showing different fragments (50 pg/µL methanol) in positive electrospray mode.

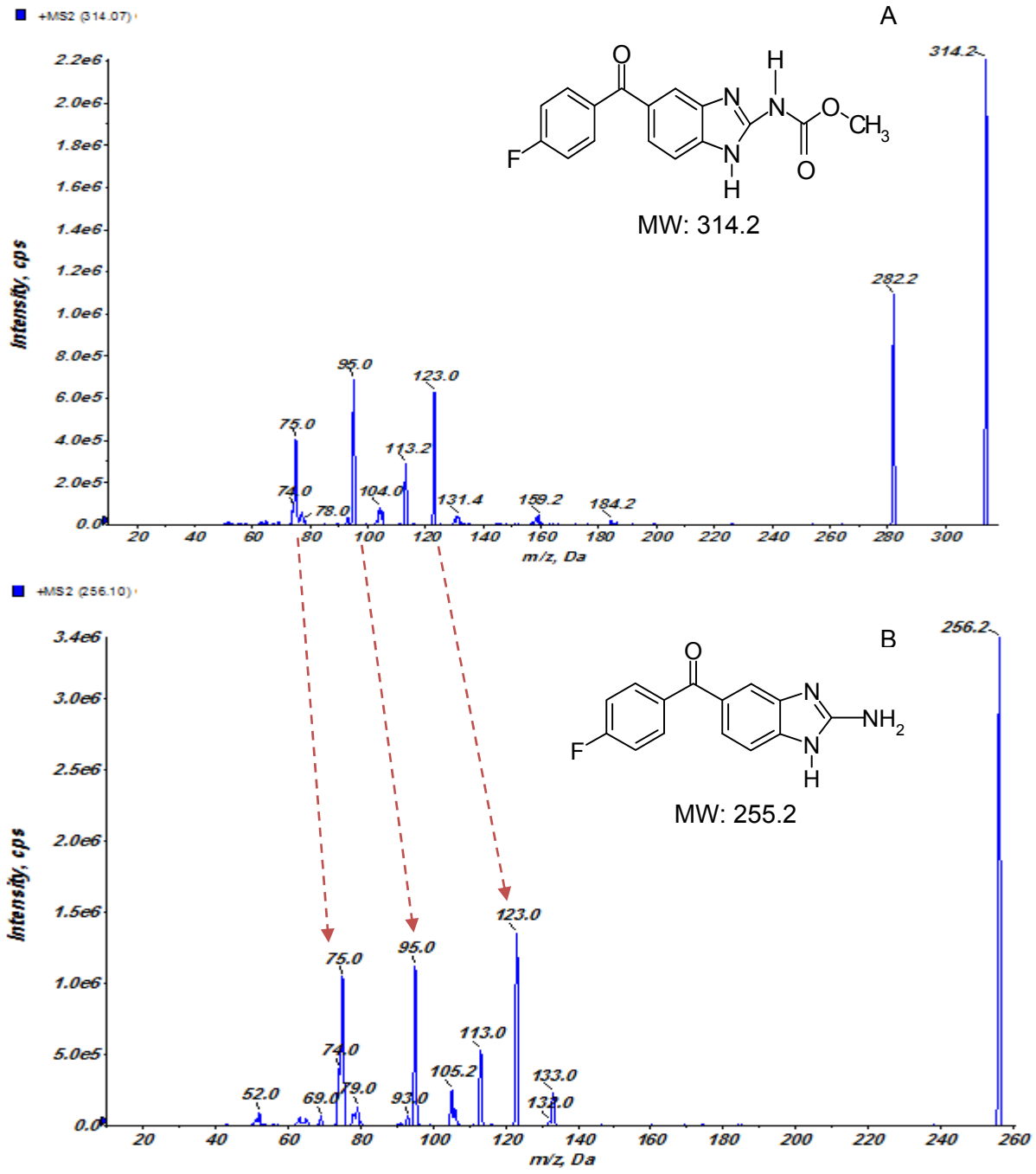


Figure 4.36: Product ion spectra of A: flubendazole, B: flubendazole metabolite (FLU-M2) in positive electrospray ionization mode showing nearly the same fragmentation patterns.

When formic acid was added to the mobile phase at different amounts, it was found that 0.01 % of formic acid was the most efficient one for sensitivity enhancement (**Figure 4.37**). However, insufficient resolution was obtained. Hence, it was necessary to re-optimize the chromatographic separation using 0.01 % of formic acid, where under these conditions higher ionization efficiency can be obtained as well as no adducts will be formed.

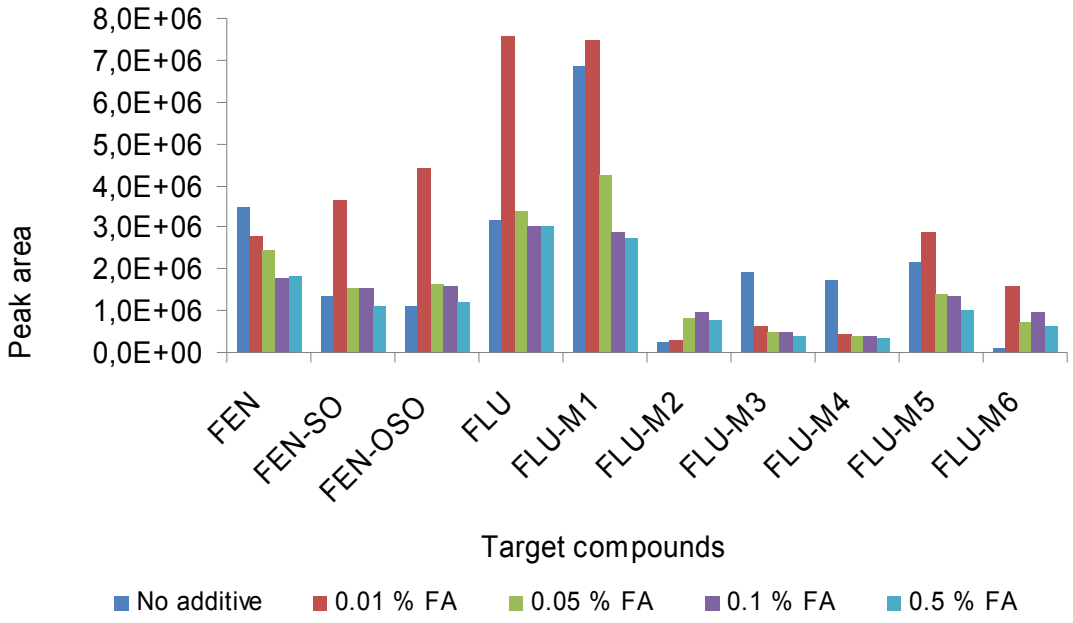


Figure 4.37: Effects of formic acid (FA) addition to the mobile phase at different concentrations on the ionisation efficiency of fenbendazole, flubendazole and their corresponding metabolites (100 pg/µL) in positive electrospray ionization mode.

Various mobile phases consisting of methanol or acetonitrile and water with formic acid were tested to obtain the maximum signal intensities during LC/MS/MS analysis at highest separation capacity. The effects of the mobile phase composition on the target compound separation and ionization efficiency were identified using 0.01% of formic acid as mobile phase additive when acetonitrile was applied instead of methanol (**Figure 4.38)**. Moreover, to determine the optimum LC flow rate, the peak area of the target compounds were measured at flow rate 1 and 0.5 mL/min. A sensitivity decrease of approximately 50 % was observed at higher flow rate. However, separation of all the target compounds with narrow peak width and relatively faster run times in addition a sufficient sensitivity to detect the target compounds al low concentrations were achieved using 1 mL/min**.** Finally, 7 µL were selected as the optimum injection volume to avoid peak fronting or peak broadening.

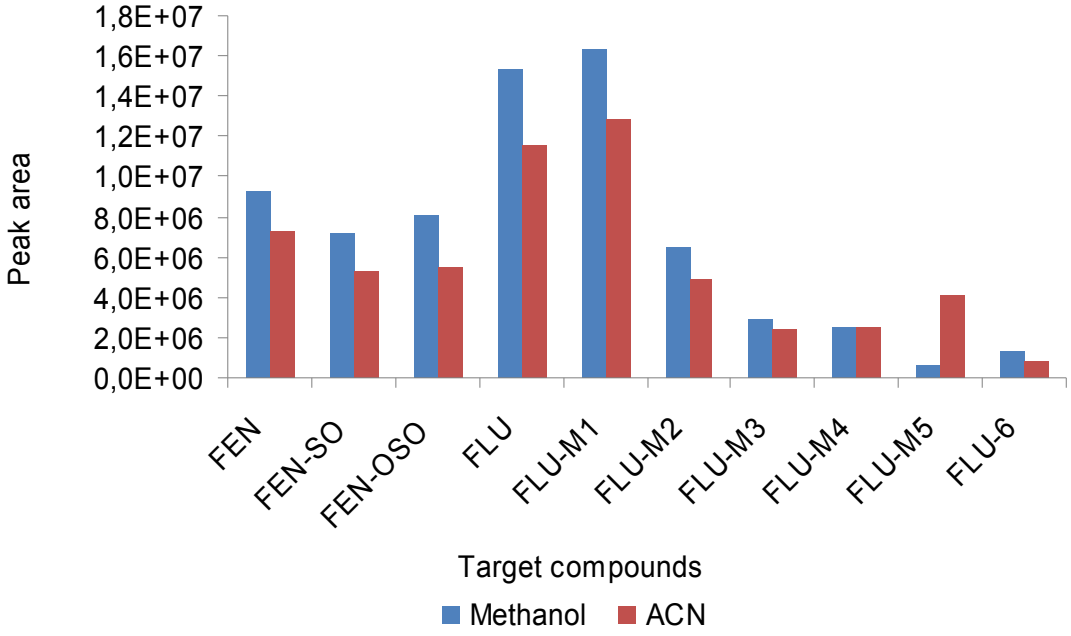
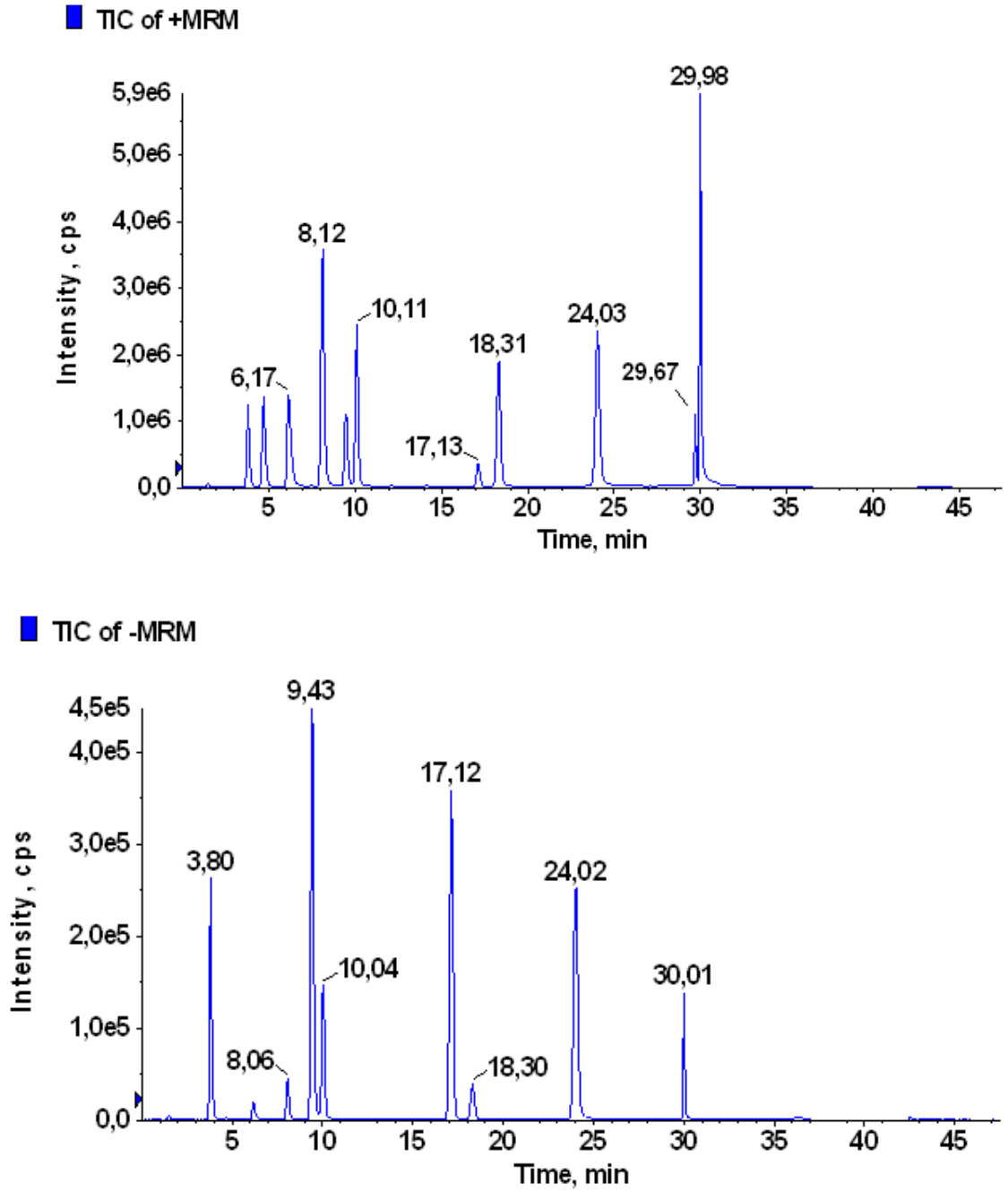


Figure 4.38: Effect of mobile phase composition (methanol versus acetonitrile) on the ionisation efficiency of fenbendazole, flubendazole and their corresponding metabolites using 0.01% of formic acid as additive (100 pg/µL).

The optimum operational conditions were achieved using mobile phase A which consisted of 10 % acetonitrile in water (v/v) with 0.01% formic acid while phase B consisted of pure acetonitrile. Reasons were the following: Ten analytes were ionized with highest ionization efficiency. Acetonitrile with formic acid gave full chromatographic separation for all the target compounds. Based on the mentioned chromatographic conditions and compounds dependent parameters, the ion source parameters were optimized and finally MRM method was created. **Figures 4.39** to **4.41** exhibit full separation for all target compounds using MRM/ESI in positive and negative ionization modes. A wide gradient from 10 % to 100% acetonitrile were required to achieve full chromatographic separation due to the wide range of polarity in the target compounds. Therefore, FEN-SO and FLU-M1, having the same precursor ion (315 m/z) were successfully identified not only based on the MRM transitions but also on their retention time (RT)**.** Several samples were analyzed by LC/MS/MS using ESI and APCI and it was found that ESI ionization was more sensitive for the analysis of fenbendazole and flubendazole with corresponding metabolites (**Figure 4.42**). Additionally, APCI chromatograms were recorded in positive and negative ion modes (**Figure 4.43)**



FLU-M3 (RT, 3.81), FLU-M4 (RT, 4.72), FLU-M2 (RT, 6.17), FLU-M1 (RT, 8.06), FLU-M5 (RT, 9.48), FEN-SO (RT, 10.11), FLU-M6 (RT, 17.13), FEN-OSO (RT,18.31), FLU (RT, 24.03), IS (RT, 29.68), FEN (RT, 29.98)

Figure 4.39: Total ion current chromatogram of fenbendazole and flubendazole with corresponding metabolites in positive mode and negative modes using electrospray ionization source.

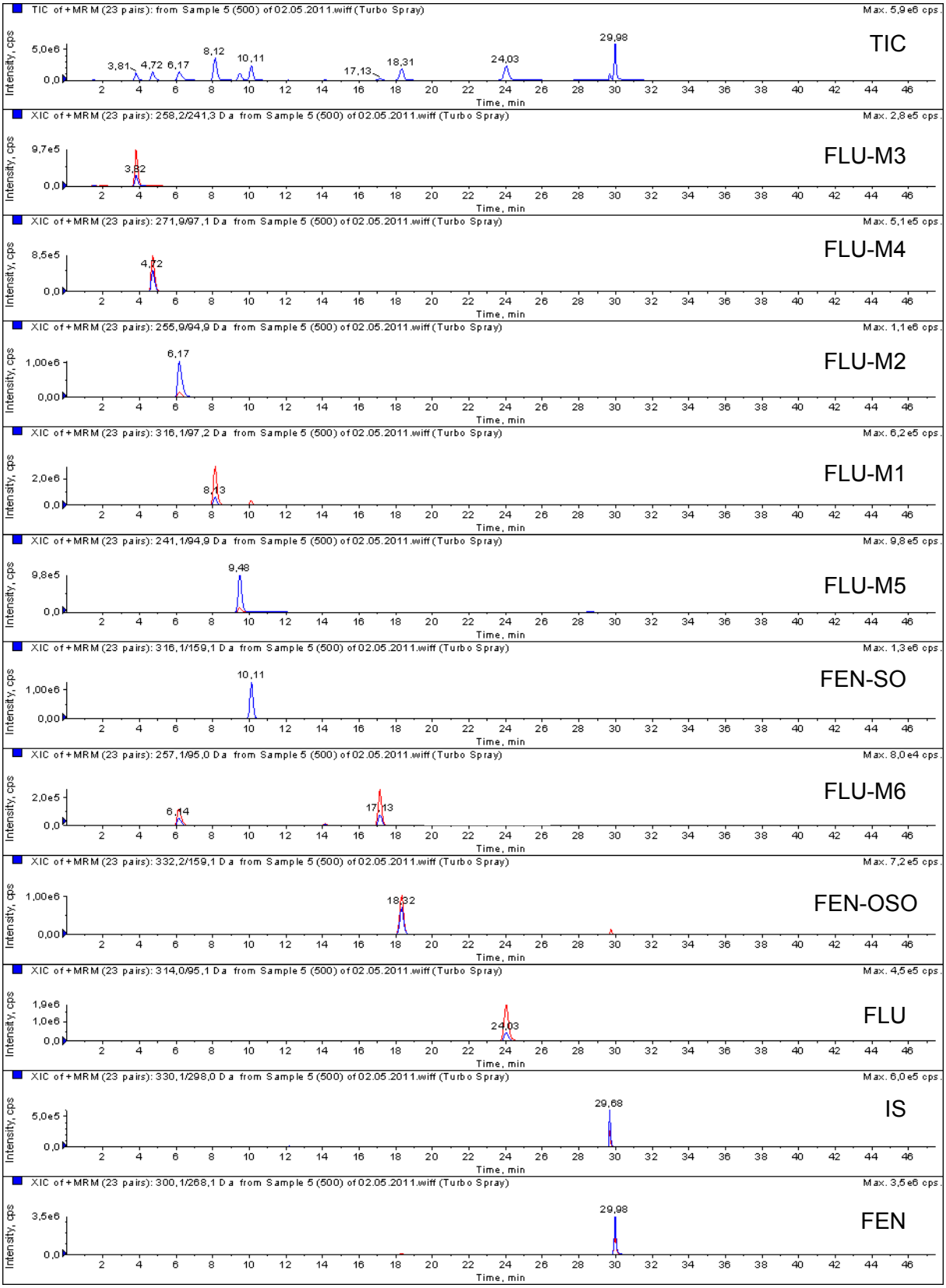


Figure 4.40: Extracted ion chromatograms of fenbendazole and flubendazole with corresponding metabolites (500 pg/µL methanol) using electrospray ionization in positive ion mode.

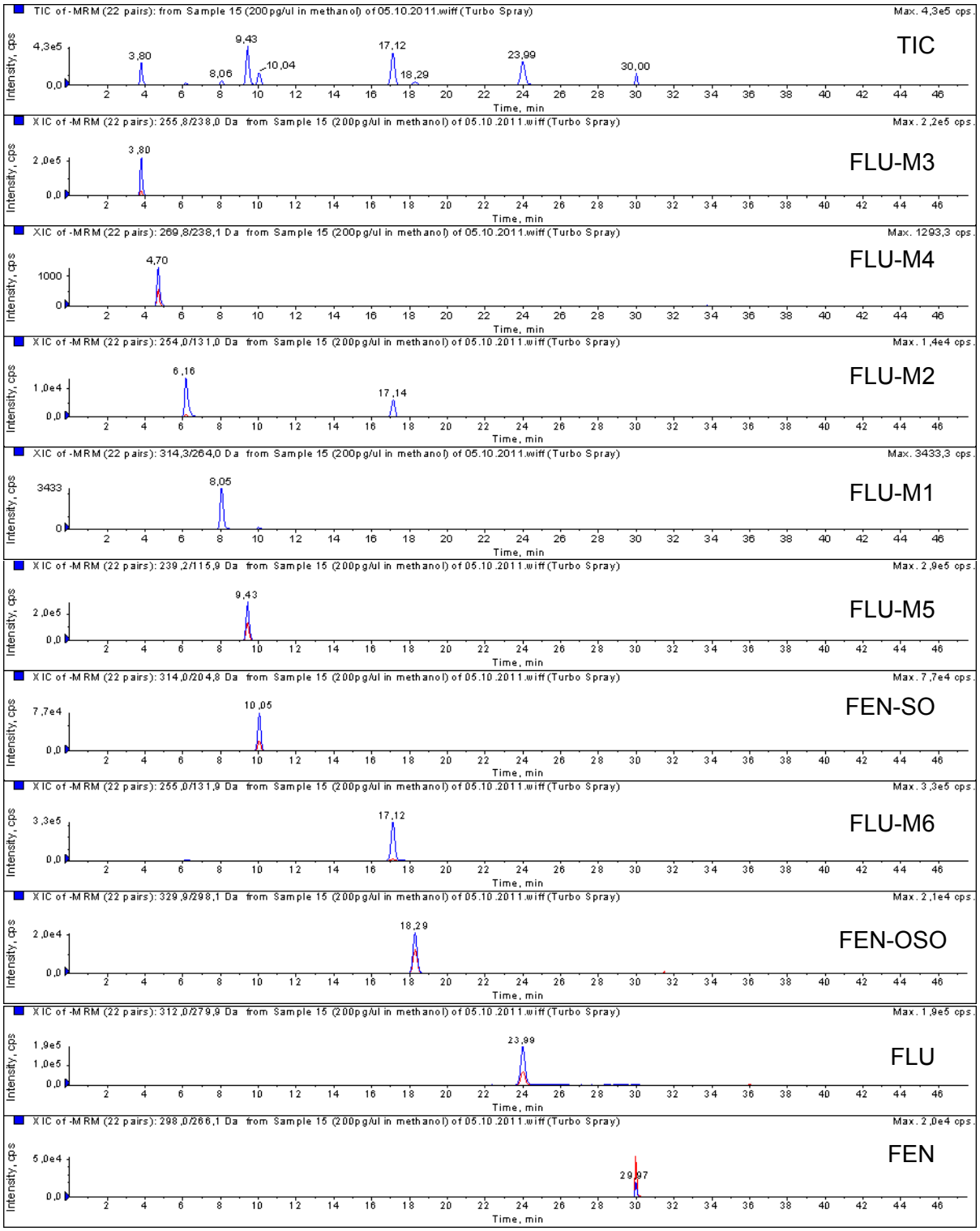


Figure 3.41: Extracted ion chromatograms of fenbendazole and flubendazole with corresponding metabolites (200 pg/µL methanol) using electrospray ionization in negative ion mode.

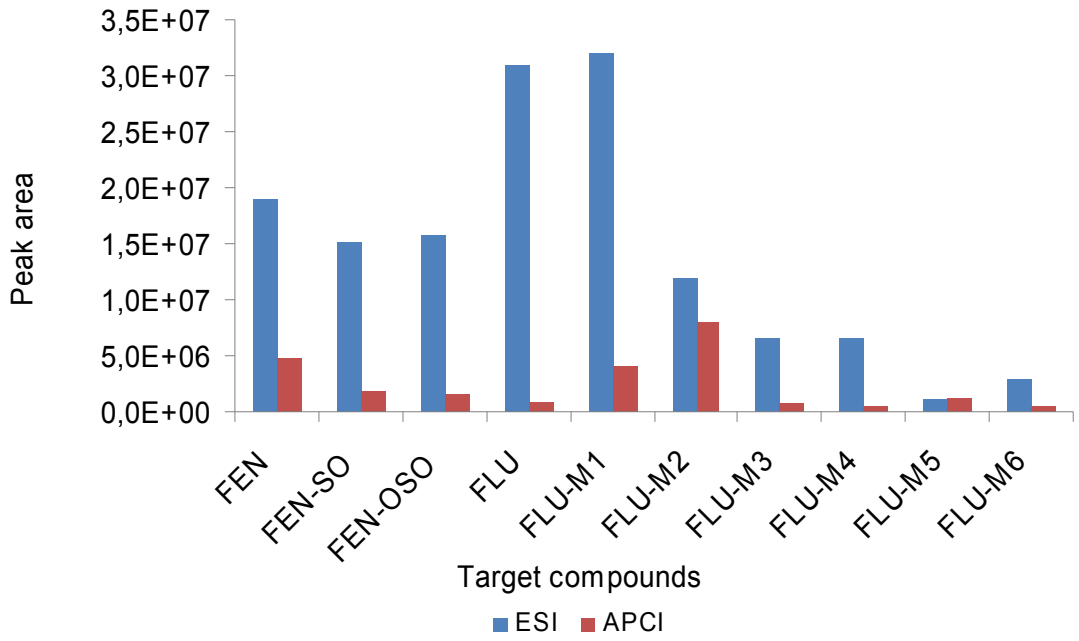
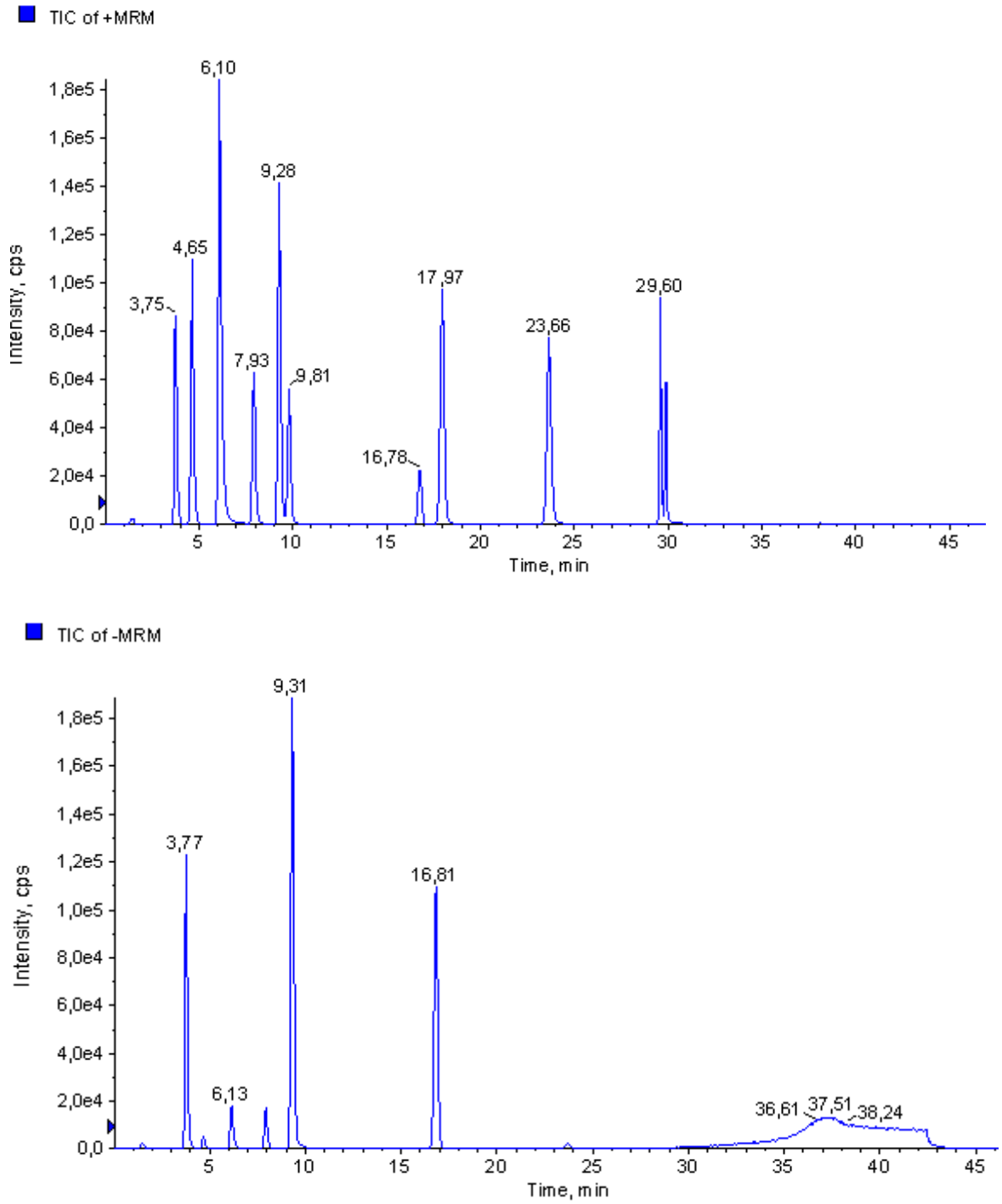


Figure 3.42: Signal intensities of electrospray (ESI) and atmospheric pressure chemical ionization (APCI) at 500-pg/µL standard mixture of fenbendazole and flubendazole with corresponding metabolites in methanol.

An explanation of the fragmentation route for the various compound molecules into the product ions, as summarised in **Table 3.6** and **3.7**, are suggested as the following: fragmentation of FLU and FEN with corresponding metabolites in the 4000 QTRAP LC/MS/MS system exhibited characteristic fragmentation with the ESI (+) source. The fragmentation process of the investigated FEN and its metabolites produces a fragment with m/z 159 which is characteristic for FEN and its metabolites. The latter is corresponding to $[M\text{-}OCH_3\text{-}SC_6H_5]^+$, $[M\text{-}OCH_3\text{-}SOC_6H_5]^+$ or $[M\text{-}OCH_3\text{-}S_2OC_6H_5]^+$ of FEN, FEN-SO and FEN-OSO, respectively. The other fragments with m/z 268.1, 300.0 and 191.0 were obtained by loss of $–OCH_3$ form the chemical structure of FEN, FEN-OSO and $[MSOC_6H_5]^+$ for FEN-SO, respectively. The common fragments of FLU and its metabolites were m/z 123 and 95. These are corresponding to OC_7H_4F and C_6H_4F respectively. The other fragments with m/z, 282 and 284.2 were obtained by the loss of $–OCH_3$ from FLU and FLU-M1, m/z 240.2 and m/z 134.2 of FLU-M3 originated from $[M–NH_3]^+$ and $[M\text{-}OC_7H_4F]^+$. The fragment with m/z 148.1 originated from $[M\text{-}OC_7H_4F]^+$ of FLU-M4, while m/z 97.2 originated from [M–174 Da]. It should be noted that the suggested fragmentation patterns of FEN, FEN-SO and FLU are in agreement with those suggested by De Ruyck et al., (2002).



FLU-M3 (RT, 3.75), FLU-M4 (RT, 4.65), FLU-M2 (RT, 6.10), FLU-M1 (RT, 7.93), FLU-M5 (RT, 9.28), FEN-SO (RT, 9.81), FLU-M6 (RT, 16.78), FEN-OSO (RT,17.97), FLU (RT, 23.66), IS (RT, 29.60), FEN (RT, 29.90)

Figure 4.43: Total ion current chromatograms of fenbendazole and flubendazole with corresponding metabolites in positive mode and negative modes (200 pg/µL methanol) using atmospheric pressure chemical ionization source (APCI).

4.5 Method validation

4.5.1 Recovery tests

In surface water samples, absolute recoveries were determined by analyzing spiked surface water samples at 5 and 25 µg/L (n = 7 and 4, respectively) using SDB 1 cartridges and HPLC/UVD instruments. In LC/MS/MS water samples were spiked with the target compounds at 0.1, 0.2 and 1 µg/L, respectively. The typical recoveries in surface water samples ranged from 84 % to 118 % using either HPLC/UVD or LC/MS/MS (**Table 4.10).**
Relative recoveries were calculated for manure, soil and manured soil. For manure samples, the range of percentage recoveries for all of the target compounds was 78 to 116 %, 76 to 106 % and 94 to 118 % with RSD ≤ 19, 14 and 12 %, respectively. Here, manure samples were extracted directly using ethyl acetate at pH 9.5 or after lyophilization using methanol or methanol/ethyl acetate mixture (1:4, v/v) at alkaline pH via USE, respectively. The recovery marginally fell below the acceptable recovery rate once, i.e., 67 % with RSD = 4 % for fenbendazole at 50 µg/kg extracted via USE.
The mean recoveries of the target compounds spiked to soil samples and extracted using methanol/ethyl acetate mixture (1:4, v/v) at alkaline pH ranged from 70 to 104 % and 83 to 99 % with RSD < 21 % for sand and clay soil samples, respectively. At 4 µg/kg spiking level in sand soil samples, the recoveries of FEN and FLU-M5 were 56 and 120 % with RSD < 13 and 9 %. Near MQL, those values are acceptable as well (EC, 2002). In the manured soil samples directly extracted using methanol/ethyl acetate mixture (1:4, v/v) at alkaline pH, the recoveries ranged from 84 to 116 % and 81 to 103 % with RSD ≤ 20 and 10 %. After USE, mean recoveries were 86 to 119 % and 88 to 112 %, respectively. These values were calculated for the target compounds individually with at least three different spiking levels and 3 to 4 replicates for each. 7 replicates were analyzed only at 2 µg/kg for of manure, sand soil and manured sand soil samples directly solvent extracted**.**
In general the calculated recoveries in this study matched those of already published data. For example, the obtained recovery range of FEN, FEN-SO, FEN-OSO and FEN-OH spiked in milk samples was 83 to 91 % with RSD < 6 % (Fletouris et al., 1996). Dowling et al. (2005) reported that 27 to 92 % of 12 benzimidazole compounds including, FEN, FEN-SO, FEN-OSO, FLU and FLU-M2, spiked in liver samples using ethyl acetate extraction at alkaline pH were recovered. In water samples, Van De Steene and Lambert (2008) determined 9 basic pharmaceuticals including FLU in wastewater and surface water samples, where the recoveries ranged from 90 to 105 %. Balizs (1999) extracted a mixture of 15 benzimidazoles, including FEN, FEN-SO, FEN-OSO and FLU, spiked in pig muscle tissue. As clean-up procedure, the samples were extracted by ethyl acetate and then

followed by solid-phase extraction on SDB1 cartridges. The obtained recoveries of the majority of the target compounds were below 60 %.
In the present work, however, there was no relevant difference between the obtained recoveries at low and high spiking levels for all of the target compounds in the different sample matrices under study.

4.5.2 Accuracy and precision test

All correlation coefficients of solvent calibration curves at different levels were >0.995, reflecting high linearity and precision of the substance specific responses. To validate the precision of the proposed methods, repeatability and intermediate precision were examined for the target compounds in surface water, manure, soil and manured soil. In surface water samples using SDB1 cartridges and LC/MS/MS at pH 2.3, repeatability and intermediate precision were usually found less than 20 % for the target compounds spiked at 0.1, 0.2 and 1 µg/L in water samples (**Table 4.11).** In this test series, 3-4 replicates for each concentration were measured in one day and repeated for two other days. Due to the time consuming manure and soil analysis, 3 spiking levels (n= 3-4) of the target compounds were used to test these parameters. Only in manured clay soil samples treated by direct solvent extraction or USE, these parameters were tested at 2 and 100 µg/kg. Each spiking level (n=4) was analyzed in separate days and the repeatability was calculated. Intermediate precision was calculated as average of RSD % values of the calculated repeatability in these 3 days.
RSD % ≤ 19, 16 and 12 % were gained for manure sample after direct ethyl acetate extraction at alkaline pH, after lyophilization and direct methanol extraction of the manure solids and after USE using methanol/ethyl acetate mixture at alkaline pH, respectively. Except of FEN in case of the sample extracted via USE technique and spiked with 50 µg/kg, where RSD was 27 % (**Table 4.12** to **4.14**). For the soil samples extracted using methanol/ethyl acetate mixture at alkaline pH, RSD % were ≤ 21 % for all target compounds spiked in sand soil and 15 % in clay soil samples (**Table 4.15** and **4.16**). In the manured sand and clay soil samples directly extracted using methanol/ethyl acetate mixture at alkaline pH, RSD % were ≤ 20 and 10 %, respectively. After USE, RSD were 16 and 17 %, respectively. Only at 2 µg/kg, RSD were 25 % for FEN and 23 % for FLU-M2 (**Table 4.17** to **4.19**).
Method accuracy was calculated as relative error bias [accuracy (%) = 100 x (($C_{spiked} - C_{determined}$)/$C_{spiked}$)] at different spiking levels. The accuracy of the method was studied by analyzing zero samples from different sample matrices spiked at 3 levels in 3 to 4 replicates. In general, accuracy for most of the target compounds in water, manure, soil and manured

soil were less than ≤ 25% except of FEN metabolite and FLU with corresponding metabolites, FEN at 50 µg/kg in liquid manure samples extracted via ethyl acetate and FLU-M2 at 2 µg/kg in sand soil sample. These results definitely confirm that the developed method is of high precision and accuracy enabling the analytical determination of FEN and FLU with corresponding metabolites in complex sample matrices at low µg/kg.

Table 4.10: Recovery rates of fenbendazole and flubendazole with corresponding metabolites spiked in surface water samples extracted using styrene-divinylbenzene cartridges at pH 2.3 and finally analyzed using HPLC/UVD and LC/MS/MS.

Analytes	HPLC/UVD Recovery [%] ± RSD [%]		LC/MS/MS Recovery [%] ± RSD [%]		
	5 µg/L (n =7)	25 µg/L (n =4)	0.1 µg/L (n =7)	0.2 µg/L (n =6)	1 µg/L (n =4)
FEN	99 ± 11	96 ± 10	109 ± 11	98 ± 9	84 ± 6
FEN-SO	100 ± 4	98 ± 7	111 ± 8	106 ± 11	94 ± 5
FEN-OSO	107 ± 6	99 ± 8	111 ± 6	107 ± 8	88 ± 7
FLU	97 ± 7	92 ± 5	108 ± 13	87 ± 17	84 ± 7
FLU- M1	102 ± 4	99 ± 5	104 ± 8	98 ± 5	103 ± 5
FLU-M2	100 ± 7	98 ± 5	105 ± 6	97 ± 13	105 ± 4
FLU-M3	98 ± 11	100 ± 5	111 ± 11	97 ± 9	116 ± 8
FLU-M4	107 ± 13	99 ± 4	113 ± 8	88 ± 7	118 ± 8
FLU-M5	96 ± 7	97 ± 6	105 ± 6	97 ± 8	100 ± 6
FLU-M6	95 ± 8	97 ± 7	108 ± 7	110 ± 7	102 ± 3

Table 4.11: Repeatability, intermediate precision and accuracy of the LC/MS/MS analysis of fenbendazole and flubendazole with corresponding metabolites in surface water samples.

Analytes	Repeatability			Intermediate Precision			Accuracy		
	0.1 µg/L (n =6)	0.2 µg/L (n =6)	1 µg/L (n =4)	0.1µg/L (n =16)	0.2 µg/L (n =14)	1 µg/L (n =12)	0.1 µg/L (n =16)	0.2 µg/L (n =14)	1 µg/L (n =12)
FEN	14	17	8	7	11	6	-10	-23	-15
FEN-SO	7	8	5	6	9	5	-2	-4	-9
FEN-OSO	10	11	7	7	10	6	-5	-5	-12
FLU	11	9	6	7	8	6	-9	-13	-16
FLU- M1	8	5	4	5	6	5	-4	-1	-3
FLU-M2	8	13	7	9	10	7	-6	-2	-3
FLU-M3	11	9	8	9	8	5	1	2	3
FLU-M4	8	7	9	7	6	6	-1	-3	3
FLU-M5	11	9	5	6	9	6	-5	-5	-5
FLU-M6	5	7	6	5	6	4	-1	6	-2

Table 4.12: Repeatability, intermediate precision and accuracy of the LC/MS/MS analysis of fenbendazole and flubendazole with corresponding metabolites in liquid manure samples directly extracted with ethyl acetate at pH 9.5.

Analytes	Spiked concentration [µg/kg]	Precision (repeatability) RSD [%]	Precision (intermediate) RSD [%]	Accuracy [%]
FEN	2*	19	1	-14
	10**	3		-15
	100***	9		-14
FEN-SO	2*	11	13	-6
	10**	8		11
	100***	16		-14
FEN-OSO	2*	11	9	-10
	10**	9		6
	100***	18		-6
FLU	2*	17	11	-8
	10**	4		8
	100***	8		-12
FLU-M1	2*	13	7	-4
	10**	4		-2
	100***	8		-14
FLU-M2	2*	14	12	-2
	10**	16		-22
	100***	7		-15
FLU-M3	2*	12	9	-7
	10**	2		-4
	100***	6		-18
FLU-M4	2*	17	9	-7
	10**	17		-22
	100***	8		-16
FLU-M5	2*	12	10	-1
	10**	5		4
	100***	8		-14
FLU-M6	2*	6	15	-7
	10**	11		16
	100***	8		-12

* n = 7, ** n = 4 and *** n = 3

Table 4.13: Repeatability, intermediate precision and accuracy of the LC/MS/MS analysis of fenbendazole and flubendazole with corresponding metabolites in lyophilized manure samples after direct methanol extraction at original pH.

Analytes	Spiked concentration [µg/kg] (n = 4)	Precision (repeatability) RSD [%]	Precision (intermediate) RSD [%]	Accuracy [%]
FEN	4	8	16	6
	10	12		-18
	100	8		-24
FEN-SO	4	5	10	-13
	10	8		-15
	100	4		-8
FEN-OSO	4	6	11	5
	10	9		-14
	100	6		-10
FLU	4	6	10	2
	10	10		-14
	100	6		-12
FLU-M1	4	5	10	2
	10	7		-16
	100	3		-12
FLU-M2	4	6	4	3
	10	7		-5
	100	3		-3
FLU-M3	4	5	10	2
	10	7		-17
	100	5		-4
FLU-M4	4	5	8	3
	10	7		-13
	100	4		-3
FLU-M5	4	5	11	6
	10	6		-15
	100	2		-3
FLU-M6	4	4	5	-5
	10	14		-13
	100	8		-12

Table 4.14: Repeatability, intermediate precision and accuracy of the LC/MS/MS analysis of fenbendazole and flubendazole with corresponding metabolites in lyophilized manure samples after ultrasound assisted extraction with methanol/ethyl acetate (1:4; v/v; pH 9.5).

Analytes	Spiked concentrations [µg/kg] (n = 4)	Precision (repeatability) RSD [%]	Precision (intermediate) RSD [%]	Accuracy [%]
FEN	4	6	27	18
	50	4		-33
	100	6		3
FEN-SO	4	2	1	3
	50	5		5
	100	9		9
FEN-OSO	4	3	4	8
	50	5		5
	100	12		12
FLU	4	3	6	7
	50	4		-6
	100	12		1
FLU-M1	4	3	4	-1
	50	4		6
	100	6		0
FLU-M2	4	4	3	2
	50	6		-3
	100	10		2
FLU-M3	4	11	3	4
	50	5		-2
	100	9		1
FLU-M4	4	6	4	1
	50	5		3
	100	8		-4
FLU-M5	4	5	3	4
	50	5		3
	100	9		-1
FLU-M6	4	10	6	1
	50	3		9
	100	9		-2

Table 4.15: Repeatability, intermediate precision and accuracy of the LC/MS/MS analysis of fenbendazole and flubendazole with corresponding metabolites in sand soil samples directly extracted using methanol/ethyl acetate (1:4, v/v, pH 9.5).

Analytes	Spiked concentration [µg/kg]	Precision (repeatability) RSD [%]	Precision (intermediate) RSD [%]	Accuracy [%]
FEN	2*	9	12	1
	40**	7		-17
	100***	14		4
FEN-SO	2*	9	9	-15
	40**	6		-12
	100***	8		0
FEN-OSO	2*	8	8	-15
	40**	5		-14
	100***	1		-2
FLU	2*	5	9	-14
	40**	5		-16
	100***	1		-1
FLU-M1	2*	10	10	-12
	40**	4		-14
	100***	1		3
FLU-M2	2*	21	17	-30
	40**	5		-11
	100***	10		-1
FLU-M3	2*	9	9	-12
	40**	7		-14
	100***	9		-5
FLU-M4	2*	10	10	-11
	40**	4		-15
	100***	15		3
FLU-M5	2*	7	6	1
	40**	8		-19
	100***	7		-2
FLU-M6	2*	11	11	-17
	40**	5		-11
	100***	5		2

* n = 7, ** n = 4 and *** n = 3

Table 4.16: Repeatability, intermediate precision and accuracy of the LC/MS/MS analysis of fenbendazole and flubendazole with corresponding metabolites in clay soil samples directly extracted using methanol/ethyl acetate (1:4, v/v, pH 9.5).

Analytes	Spiked concentration (µg/kg) (n = 4)	Precision (repeatability) RSD [%]	Precision (intermediate) RSD [%]	Accuracy [%]
FEN	4	13	2	- 3
	40	8		- 1
	100	3		- 1
FEN-SO	4	3	2	- 7
	40	5		- 4
	100	5		- 6
FEN-OSO	4	2	3	- 1
	40	5		- 4
	100	3		- 6
FLU	4	4	1	- 1
	40	6		- 3
	100	2		- 3
FLU-M1	4	4	6	- 14
	40	4		- 4
	100	3		- 7
FLU-M2	4	5	4	- 13
	40	3		-10
	100	1		- 5
FLU-M3	4	3	5	- 17
	40	6		- 9
	100	7		- 13
FLU-M4	4	7	1	- 17
	40	15		- 17
	100	8		- 15
FLU-M5	4	9	3	- 15
	40	6		- 10
	100	8		- 11
FLU-M6	4	4	2	- 6
	40	7		- 8
	100	4		- 9

Table 4.17: Repeatability, intermediate precision and accuracy of the LC/MS/MS analysis of fenbendazole and flubendazole with corresponding metabolites in manured sand soil samples directly extracted using methanol/ethyl acetate (1:4, v/v, pH 9.5).

Analytes	Spiked concentration [µg/kg] (n = 4)	Precision (repeatability) RSD [%]	Precision (intermediate) RSD [%]	Accuracy [%]
FEN	2*	25	13	16
	10	8		-6
	100	12		-7
FEN-SO	2*	18	11	5
	10	6		3
	100	7		-14
FEN-OSO	2*	19	12	12
	10	6		3
	100	7		-12
FLU	2*	19	11	15
	10	4		-4
	100	7		-6
FLU-M1	2*	17	10	7
	10	4		-1
	100	8		-3
FLU-M2	2*	18	11	10
	10	5		-2
	100	8		-12
FLU-M3	2*	18	8	0
	10	8		-6
	100	5		-15
FLU-M4	2*	16	8	-2
	10	4		-7
	100	7		-14
FLU-M5	2*	18	9	2
	10	9		12
	100	7		-12
FLU-M6	2*	20	14	13
	40	16		10
	100	12		-13

* n = 7

Table 4.18: Repeatability, intermediate precision and accuracy of the LC/MS/MS analysis of fenbendazole and flubendazole with corresponding metabolites in manured clay soil samples directly extracted using methanol/ethyl acetate (1:4, v/v, pH 9.5).

Analytes	Spiked concentration (µg/kg)	Precision (repeatability) RSD [%]	Precision (intermediate) RSD [%]	Accuracy [%]
FEN	2	7	17	-19
	100	4		3
FEN-SO	2	3	2	-11
	100	5		-8
FEN-OSO	2	3	11	-15
	100	7		1
FLU	2	5	5	-9
	100	5		-3
FLU-M1	2	2	6	-16
	100	6		-8
FLU-M2	2	6	5	-10
	100	4		-3
FLU-M3	2	7	8	-15
	100	10		-5
FLU-M4	2	4	1	-13
	100	6		-12
FLU-M5	2	6	5	-13
	100	6		-5
FLU-M6	2	9	10	-11
	100	5		3

Table 4.19: The repeatability, intermediate precision and accuracy of the LC/MS/MS analysis fenbendazole and flubendazole with corresponding metabolites in manured sand soil samples ultrasound assisted extracted using methanol/ethyl acetate (1:4, v/v, pH 9.5).

Analytes	Spiked concentration [µg/kg] (n = 4)	Precision (repeatability) RSD [%]	Precision (intermediate) RSD [%]	Accuracy [%]
FEN	2	6	9	1
	10	16		9
	100	5		-9
FEN-SO	2	5	1	-8
	10	5		-7
	100	6		-7
FEN-OSO	2	7	4	-7
	10	6		-2
	100	5		-9
FLU	2	6	4	-4
	10	3		-9
	100	4		-11
FLU-M1	2	8	3	-11
	10	3		-7
	100	3		-12
FLU-M2	2	23	11	7
	10	11		19
	100	8		-4
FLU-M3	2	2	1	-9
	10	3		-7
	100	6		-8
FLU-M4	2	9	1	-6
	10	3		-4
	100	5		-6
FLU-M5	2	10	8	-7
	10	7		-14
	100	5		-10
FLU-M6	2	9	6	-1
	40	7		5
	100	7		-7

Table 4.20: The repeatability, intermediate precision and accuracy of the LC/MS/MS analysis fenbendazole and flubendazole with corresponding metabolites in manured clay soil samples ultrasound assisted extracted using methanol/ethyl acetate (1:4, v/v, pH 9.5).

Analytes	Spiked concentration (µg/kg) (n = 4)	Precision (repeatability) RSD [%]	Precision (intermediate) RSD [%]	Accuracy [%]
FEN	2	17	9	7
	50	12		-6
FEN-SO	2	9	10	3
	50	2		-10
FEN-OSO	2	13	7	4
	50	4		-6
FLU	2	13	15	12
	50	5		-10
FLU-M1	2	9	2	-2
	50	4		-8
FLU-M2	2	8	2	-3
	50	14		-6
FLU-M3	2	9	1	-3
	50	7		-2
FLU-M4	2	8	6	3
	50	14		-6
FLU-M5	2	6	4	1
	50	3		-6
FLU-M6	2	9	13	6
	50	7		-12

4.5.3 Method detection and quantitation limits

The sensitivity of the analytical method was measured using the instrumental detection (IDL) and quantitation (IQL) limits as well as method detection (MDL) and quantitation limits (MQL). Two approaches were used in this study to calculate detection and quantitation limits. First, IDL and IQL were defined on signal to noise ratios of S/N >3 or S/N >10. The IDL and IQL values for the target compounds thus ranged from 0.7 to 4 and 2 to 14 pg (absolute concentration), respectively. For FLU-M6, however, these values were 14 and 42 pg (**Table 4.21**). Additionally, the MDL and MQL of individual compounds in surface water, manure and manured soil were determined by calculating the standard deviation at the 99-% confidence level of 7 replicates spiked at a concentration close to the expected IDL, e.g., 5 to 0.1 ng/L in surface water for HPLC-UVD and LC/MS/MS analysis and 2 µg/kg for manure, soil and manured soil for LC/MS/MS analysis. The concentrations of FEN and FLU-M6 were half and double of the spiked concentrations applied for the other compounds, respectively. The MDL was determined by multiplying the sample standard deviation calculated from each group of fortified samples by the Student's t-variate a one sided t-test at the 99% confidence level with n−1 degrees of freedom.

For surface water samples analyzed by means of HPLC/UVD, MDL and MQL, values of the target compounds were < 2 and < 6.5 µg/L, while samples analyzed by means of LC/MS/MS these values ranged from 0.015 to 0.04 µg/L and 0.03 to 0.12 µg/L, respectively (**Table 4.22**). From the results of surface water analysis, it became obvious that LC/MS/MS was up to 160 times more sensitive than HPLC/UVD. Therefore, the analytical solutions of the more complex sample matrices, i.e., liquid pig manure, soil and manured soil samples, were exclusively analyzed by means of LC/MS/MS. MDL and MQL of the target compounds were ≤ 1 and 3.1 µg/kg in manure samples directly extracted using ethyl acetate at pH 9.5. These values are satisfactory for analyzing the target compounds in this complex matrix at low concentration levels (**Table 4.21**).

With the exception of FLU-M6, Similar analytical sensitivity was achieved in sand soil and manured sand soil samples where the MDL and MQL for most of target compounds were < 1.5 and 4.4 µg/kg, respectively. For FLU-M6 in sand and manured sand soil samples, MDL and MQL were ≤ 2.5 and 7.9 µg/kg, respectively. Those higher MDL and MQL for this compound corresponded to its low response in ESI+ mode. This may be attributed to its potential decomposition in the ion source where the heater temperature was adjusted at 600 ºC (**Table 4.23** and **4.24**). Moreover, these results matched with method detection limits determined by other researchers for some of these compounds individually or in a mixture with other benzimidazoles in different matrices.

For example, Sørensen and Hansen (1998) determined the MQL as the average results plus three times the standard deviation (SD) of the 20 measurements of muscle samples spiked

at 2 µg/kg. The MQL were calculated as the average results plus six times the SD of the 20 measurements. In their study MDLs in trout samples were 4.0, 4.5 and 3.8 µg/kg for FEN, FEN-SO and FEN-OSO respectively. The calculated MDL and MQL by Balizs (1999) for 15 benzimidazoles including FEN, FEN-SO and FEN-OSO and FLU spiked in muscle tissues of cattle and sheep were ≤ 6 and 10 µg/kg, respectively. In the same study very high values were obtained for FLU analyzed in egg sample by LC/MS/MS, where MDL and MQL values were 32 and 48 µg/kg, respectively. De Ruyck et al. (2001) calculated MDL and MQL for FLU, FLU-M1 and FLU-M2 in egg and muscle samples extracted using ethyl acetate at alkaline pH and analyzed by LC/MS/MS. These values were, respectively, 0.1 to 1 and 1 to 2 µg/kg. Taking into account the complexity of the matrices in the current study in addition to this mixture of target compounds with wide range of physicochemical properties, the developed method is sophisticated enough to determine FEN and FLU with corresponding metabolites in complex sample matrices.

Table 4.21: Instrumental detection and quantitation limits as well as method detection and quantitation limits of fenbendazole and flubendazole with corresponding metabolites in liquid manure samples directly ethyl acetate at pH 9.5 and analyzed by LC/MS/MS according EPA, (1984) and Wisconsin Department of Natural Resources (1996).

Analytes	MRM Transitions [amu]	Spiked concen -tration [µg/kg]	R ± RSD (n =7) [%]	SD	MDL [µg/kg]	MQL [µg/kg]	IDL [pg/injection]	IQL
FEN	300.1-268.1 300.1-159.1	1	86 ± 19	0.16	0.50	1.6	0.7	2.0
FEN-SO	316.1-59.1 316.1-191.1	2	94 ± 11	0.20	0.60	2.0	1.5	3.5
FEN-OSO	332.1-300.1 332.1-159.1	2	90 ± 11	0.19	0.60	1.9	3.0	8.5
FLU	314.2-282.2 314.2-95.1	2	92 ± 17	0.30	0.95	3.0	0.7	2.0
FLU-M1	256.0-95.0 256.0-123.1	2	96 ± 13	0.26	0.80	2.6	0.7	2.0
FLU-M2	241.1-95.0 241.1-123.1	2	98 ± 14	0.28	0.90	2.8	3.0	8.5
FLU-M3	257.0-95.0 257.0-123.1	2	83 ± 12	0.21	0.64	2.1	3.0	8.5
FLU-M4	316.2-284.2 316.2 - 97.2	2	93 ± 17	0.30	0.99	3.1	3.0	8.5
FLU-M5	258.1-134.1 258.1-240.3	2	99 ± 12	0.23	0.74	2.3	4.0	14.0
FLU-M6	272.1-148.0 272.1-97.0	4	93 ± 6	0.11	0.50	1.6	14.0	42.0

R: Recovery; SD: Standard deviation; RSD: Relative standard deviation;

amu: atomic mass unit

IDL and IQL were calculated according to signal to noise ratio

Table 4.22: Method detection and quantitation limits of target compounds in surface water samples extracted using SDB1 cartridges at pH 2.3 and analyzed by HPLC/UVD and LC/MS/MS according to EPA (1984) and Wisconsin Department of Natural Resources (1996).

Analytes	HPLC/UVD (λ = 280 nm) Spiked concentration [5 µg/L]		LC/MS/MS (ESI+) Spiked concentration [0.1 µg/L]	
	MDL [µg/L] (n =7)	MQL [µg/L] (n =7)	MDL [µg/L] (n =7)	MQL [µg/L] (n =7)
FEN	1.6	5.2	0.02	0.05
FEN-SO	0.7	2.1	0.02	0.03
FEN-OSO	1.1	3.5	0.04	0.12
FLU	1.1	3.4	0.02	0.06
FLU-M1	0.6	2.0	0.03	0.08
FLU-M2	1.1	3.4	0.02	0.04
FLU-M3	1.7	5.5	0.03	0.08
FLU-M4	2.1	6.5	0.03	0.07
FLU-M5	2.0	3.5	0.03	0.08
FLU-M6	1.1	3.6	0.03	0.01

Table 4.23: Method detection and quantitation limits of fenbendazole and flubendazole with corresponding metabolites in sand soil samples extracted with methanol/ethyl acetate mixture (1:4, v/v) at alkaline pH and analyzed by LC/MS/MS according EPA (1984) and Wisconsin Department of Natural Resources (1996).

Analytes	MRM Transitions [amu]	Spiked concen-tration [µg/kg]	R ± RSD (n=7) [%]	SD	MDL [µg/kg]	MQL [µg/kg]
FEN	300.1 - 268.1 300.1 - 159.1	1	101 ± 9	0.29	0.25	0.8
FEN-SO	316.1 - 159.1 316.1 - 191.1	2	85 ± 9	0.33	0.40	1.5
FEN-OSO	332.1 - 300.1 332.1 - 159.1	2	85 ± 8	0.43	0.40	1.2
FLU	314.2 - 282.2 314.2 - 95.1	2	86 ± 5	0.43	0.26	0.8
FLU-M1	256.0 - 95.0 256.0 - 123.1	2	88 ± 10	0.36	0.57	1.8
FLU-M2	241.1 - 95.0 241.1 - 123.1	2	70 ± 21	0.39	0.90	2.9
FLU-M3	257.0 - 95.0 257.0 - 23.1	2	80 ± 9	0.35	0.40	1.3
FLU-M4	316.2 - 284.2 316.2 - 97.2	2	89 ± 10	0.23	0.60	1.8
FLU-M5	258.1 - 134.1 258.1 - 240.3	2	101 ± 7	0.36	0.40	1.5
FLU-M6	272.1 - 148.0 272.1 - 97.0	4	83 ± 11	0.80	1.20	3.7

R: Recoveries; SD: Standard deviation; RSD: Relative standard deviation;
amu: Atomic mass unit.

Table 4.24: Method detection and quantitation limits of fenbendazole and flubendazole with corresponding metabolites in manured sand soil samples extracted using methanol/ethyl acetate mixture (1:4, v/v) at alkaline pH and analyzed by LC/MS/MS according EPA (1984) and Wisconsin Department of Natural Resources (1996).

Analytes	MRM Transitions [amu]	Spiked concen-tration [µg/kg]	R ± RSD (n=7) [%]	SD	MDL [µg/kg]	MQL [µg/kg]
FEN	300.1 - 268.1 300.1 - 159.1	1	116 ± 25	0.29	0.9	2.9
FEN-SO	316.1 - 159.1 316.1 - 191.1	2	105 ± 18	0.33	1.1	3.3
FEN-OSO	332.1 - 300.1 332.1 - 159.1	2	112 ± 19	0.43	1.4	4.4
FLU	314.2 - 282.2 314.2 - 95.1	2	115 ± 19	0.43	1.4	4.3
FLU-M1	256.0 - 95.0 256.0 - 123.1	2	107 ± 17	0.36	1.1	3.7
FLU-M2	241.1 - 95.0 241.1 - 123.1	2	110 ± 18	0.39	1.2	3.9
FLU-M3	257.0 - 95.0 257.0 - 123.1	2	100 ± 18	0.35	1.1	3.5
FLU-M4	316.2 - 284.2 316.2 - 97.2	2	98 ± 16	0.23	0.7	2.4
FLU-M5	258.1 - 134.1 258.1 - 240.3	2	102 ± 18	0.36	1.1	3.7
FLU-M6	272.1 - 148.0 272.1 - 97.0	4	113 ± 20	0.80	2.5	7.9

R: Recoveries; SD: Standard deviation; RSD: Relative standard deviation;
amu: Atomic mass unit.

4.6 Quantitation procedures and matrix effects

Matrix effects occur because the ESI and APCI sources are highly susceptible to co-extractants in complex and heterogeneous matrices such as manure and manured soil.

These effects may result in a signal suppression or enhancement leading to erroneous results. Two mechanisms are suggested in the literature to explain matrix effects. First, those originate from the competition between the analytes and co-eluting interfering species. The ionization efficiency depends on the ionization energy and the proton affinity of the molecules present at the interface (Gosetti et al., 2010). Another hypothesis suggests that high concentrations of interfering compounds can increase or decrease the viscosity (surface tension) of the droplets produced in the ion source. These effects may enhance or reduce the ability of the analytes to reach the gas phase (Antignac et al., 2005; Gros et al., 2006; Gosetti et al., 2010).

Thus, to achieve a reliable method, matrix effects had to be compensated. Detailed studies of the matrix effects were performed to evaluate the degree of signal suppression or enhancement and sensitivity of target compounds for these effects. For this purpose, matrix effects on the target compound responses were evaluated first in surface water. Individual peak area for each compound in surface water matrix was compared with those obtained in methanol. Surface water samples showed clear matrix effects (**Table 4.25**). Enhanced signal intensities ranging was from 7 to 30 % with RSD ± 13 % at maximum for all the target compounds at 0.1 and 1 µg/L spiking levels were observed.

Table 4.25: Extraction efficiency, matrix effects and process efficiency in surface water samples.

Analytes	Extraction efficiency (Mean ± RSD, %)		Matrix effects (Mean ± RSD, %)		Process efficiency (Mean ± RSD, %)	
	0.1 µg/L (n = 8)	1 µg/L (n = 4)	0.1µg/L (n = 3)	1 µg/L (n = 3)	0.1 µg/L (n = 8)	1 µg/L (n = 4)
FEN	85 ± 6	80 ± 7	117 ± 3	120 ± 9	99 ± 6	93 ± 7
FEN-SO	93 ± 5	85 ± 5	115 ± 3	122 ± 13	107 ± 5	100 ± 5
FEN-OSO	89 ± 6	82 ± 6	111 ± 4	123 ± 11	99 ± 6	95 ± 6
FLU	98 ± 4	79 ± 8	114 ± 2	120 ± 11	101 ± 4	92 ± 8
FLU-M1	99 ± 4	91 ± 3	107 ± 4	117 ± 11	106 ± 4	102 ± 3
FLU-M2	92 ± 3	89 ± 6	114 ± 3	116 ± 10	104 ± 3	99 ± 6
FLU-M3	101 ± 6	93 ± 4	111 ± 2	121 ± 10	112 ± 6	109 ± 4
FLU-M4	96 ± 5	94 ± 5	113 ± 4	130 ± 10	108 ± 5	117 ± 5
FLU-M5	96 ± 6	86 ± 4	100 ± 4	117 ± 13	97 ± 6	96 ± 4
FLU-M6	97 ± 5	92 ± 3	115 ± 6	120 ± 11	112 ± 5	106 ± 3

Additional data from these experiments can be obtained by comparing of the peak areas of analytes spiked before extraction with those of the standard solutions to give an overall value defined as process efficiency. Process efficiency represents the combination of matrix effects and recovery of the analytes resulted from the sample extraction process (Taylor, 2005). For example, the absolute recovery of FLU-M4 was 94 %, but the process efficiency is 117%, showing a 23-% signal enhancement. Therefore, high or low process efficiency can adversely affect the method reliability as well as the method quantification limits. Based on these results which were obtained in relatively simple surface water matrix, it was necessary to evaluate the particular matrix effects in the other sample matrices, i.e., manure and manured soil samples.

In manure, blanks were analyzed first to check the back ground concentrations. Matrix effects were evaluated based on matrix-matched and external calibrations. As shown by the comparison of the slopes of both calibration curves for 10 target compounds, negligible matrix effects in manure samples directly ethyl acetate extracted were found for FEN-SO and FEN-OSO. In contrast, considerable signal suppression was observed for FLU, FLU-M2 and FLU-M6. As it can be observed in Figure 4.38, the calibration curves of these compounds recorded in manure extracts have the lower slopes, reflecting signal suppression ranged from 6, 10 and 12 %, respectively.

In order to evaluate the efficiency of an internal standard to compensate matrix effects, the chloro-analogue of FLU was added at 50 pg/µL to the standard and analytical solutions. In the recorded chromatograms, it became obvious that the peaks increased, i.e., 19 % for FLU, 12 % for FLU-M2, 11 % for FLU-M6, 24 % for FEN-OSO and 21 % for FEN-SO. Hereby, a signal enhancement was indicated. Comparing the peak areas of the internal standard itself in standard and analytical solutions, however, a 24-% ion suppression of the internal standard can be identified. Thus, the application of one single internal standard could not compensate matrix effects neither on the structural analogues FLU and metabolites nor on the other benzimidazoles under study (**Figure 4.44**). Therefore, if there are multiple analytes of diffeent polarities to be quantified multiple internal standards were required. (Lagerwerf et al., 2000; 2005). Similar situation was observed in the manure matrix extracted by methanol after lyophilization except FLU-M3 where strong signal suppression was recorded (27 %). This suppression was compensated to become 13 % when internal standard was added (**Figure 4.45**).

Otherwise, different strategies could be followed to minimize or correct the matrix effect. For minimizing the matrix effects, selective extraction, efficient clean-up procedure and improvement of the chromatographic separation as well as the dilution of sample extracts are considered as efficient methods (Antignac et al., 2005; Gros et al., 2006; Gosetti et al., 2010). This problem cannot be always completely eliminated in the practice using the above

methods. Although full chromatographic separation for all of the target compounds was achieved, two clean-up methods were used as well as dilution of the final extracts, matrix effects were still observed. Other effective and practical methods published in the literature to correct the matrix effect, e.g., using matrix-matched calibration, internal standards, e.g., structurally similar unlabeled compound or isotopically labeled standards. Unfortunately, the use of a single structurally analogue in multi-component analysis was not successful and isotopically labeled standards were not available. Therefore, using the matrix-matched calibration curve combined with the dilution of sample extracts was considered an effective alternative solution for reliable recovery calculation. Therefore, dilution of the final extract combined with full chromatographic separation of the target compounds and two clean-up steps as well as using Eq. 3.1 as modified form of matrix-matched calibration were considered as ideal conditions to achieve reliable method during the fortification experiments. To assess the impacts of matrix effects on recoveries of the target compounds in manured soil samples, spiked at 100 µg/kg, and efficiency of the proposed method (Eq. 3.1) to compensate these effects, absolute recoveries were calculated using three calibration methods. The obtained mean recoveries were 37 to 70% and 57 to 78% calculated by external calibration and Eq. 3.1, reflecting clear signal suppression for all of the target compounds. When internal standard was added, improvements for the obtained recoveries were observed but still incomparable with those obtained by Eq.3.1 (**Table 4.26**). These results show that matrix effects had relevant impacts on the reliability of LC/MS/MS applying ESI in positive ion mode. This experiment was repeated for sand soil matrix. There were no relevant differences between the recoveries calculated by matrix-matched, internal and external calibrations (**Table 4.27**). These results indicated that matrix effects were not only based on the analyte structure. They can be also caused by the sample matrix in which the compounds are analyzed.

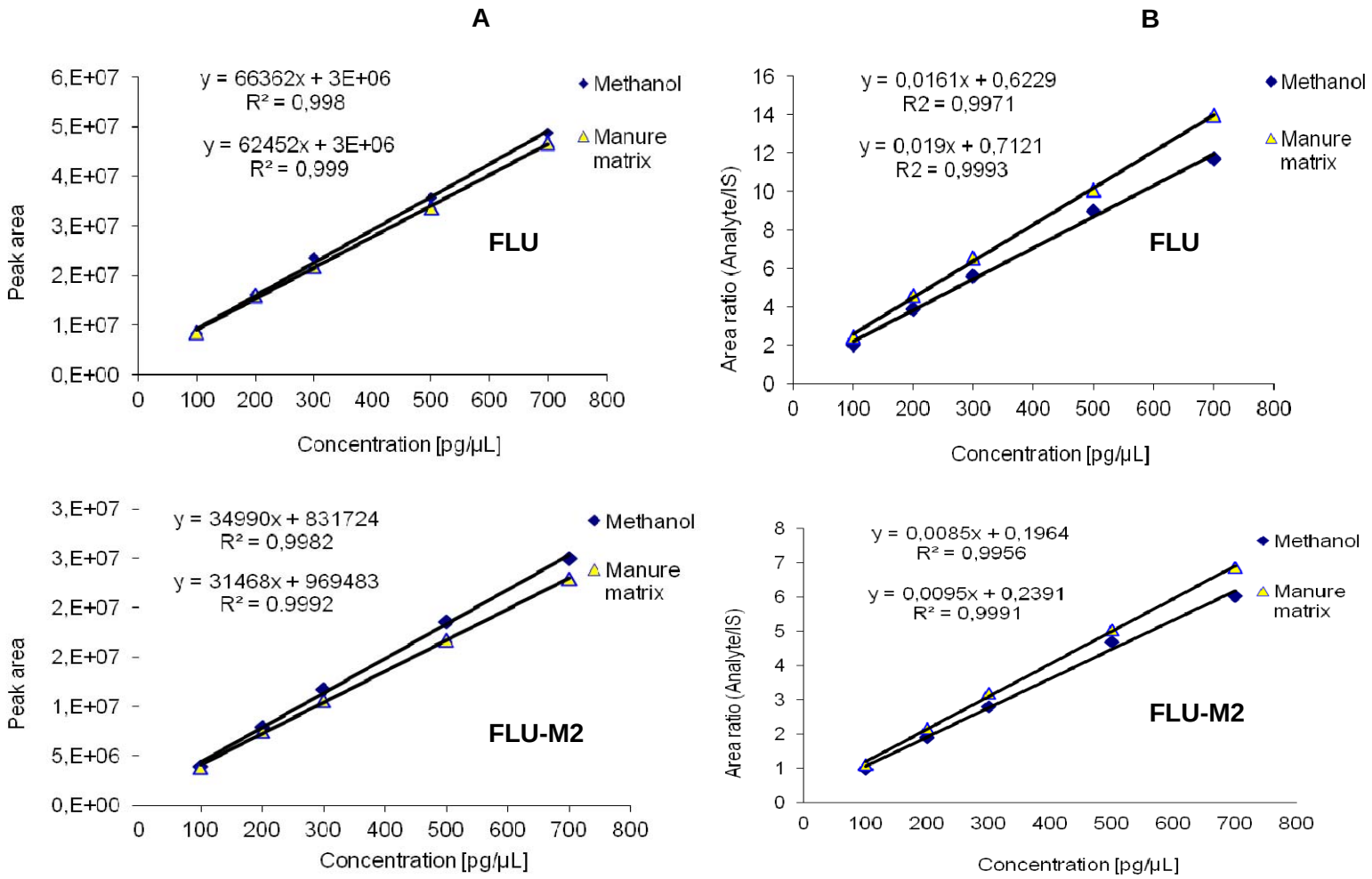
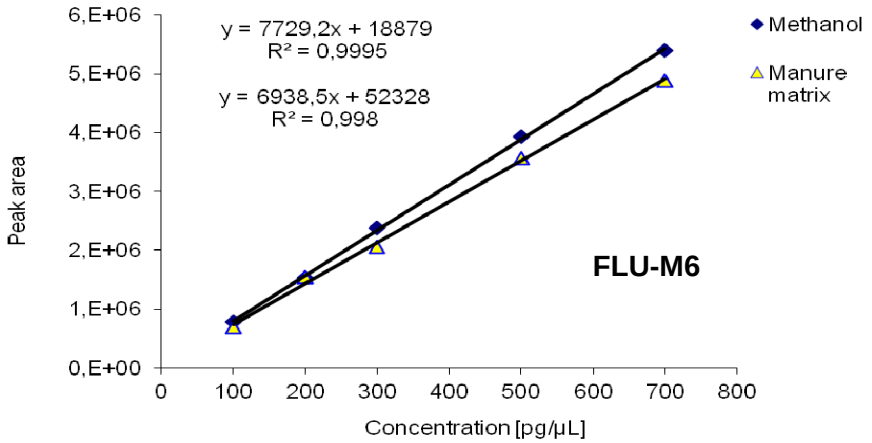
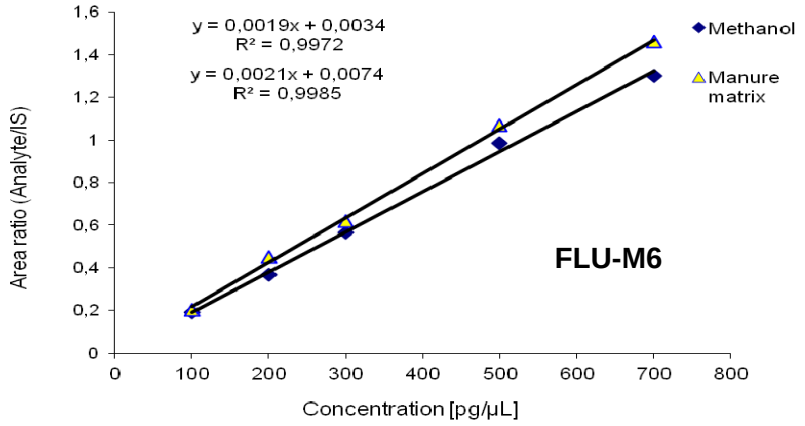
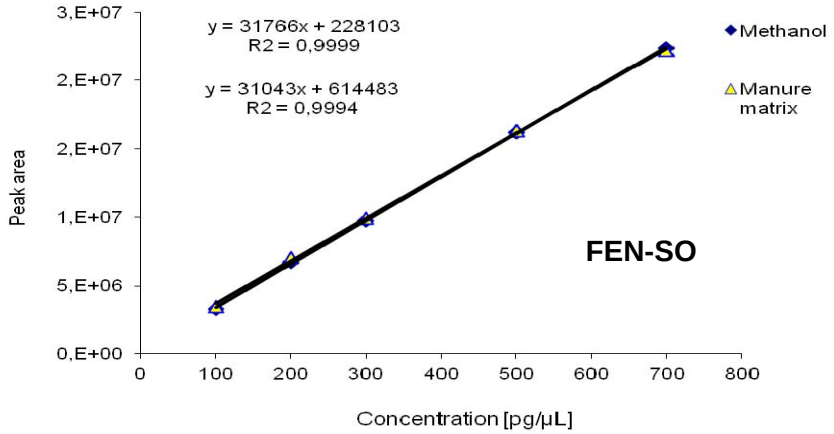


Figure 4. 44: Calibration curves for selected target compounds A: External calibrations in methanol versus those recorded in manure matrix extracted by ethyl acetate, B: Internal calibration curves in the solvent vs. those recorded in manure matrix extracted by ethyl acetate







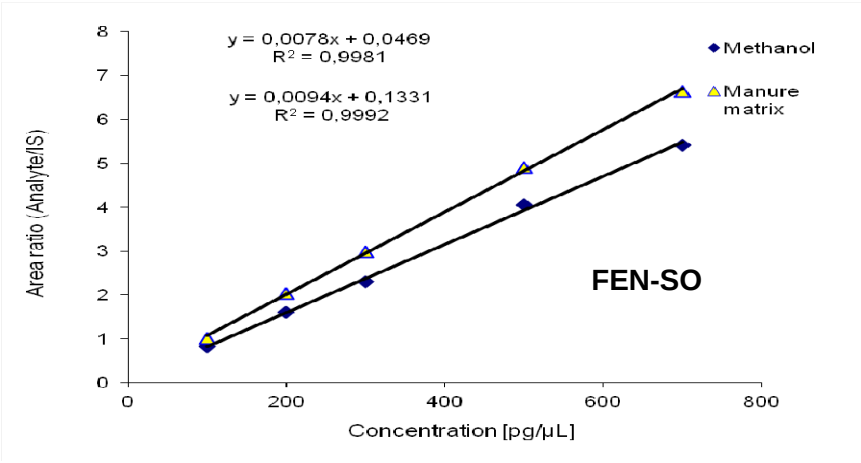
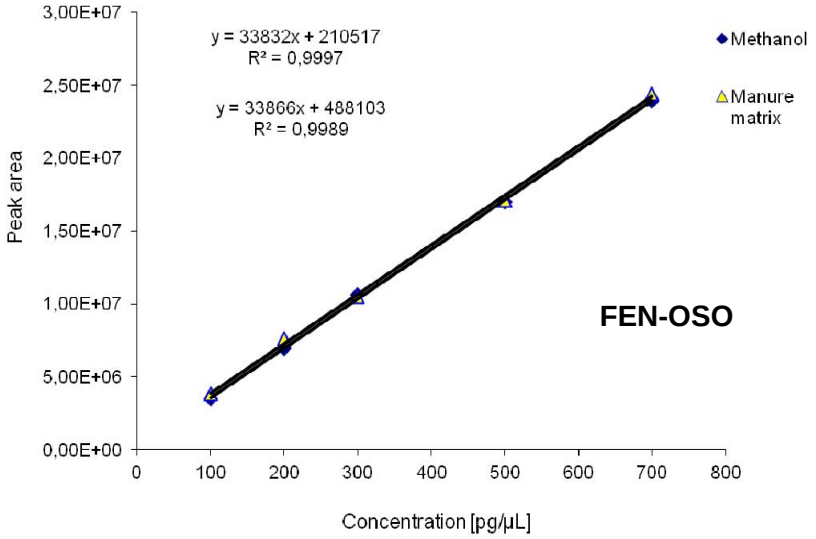


Figure 4.44: Continued.



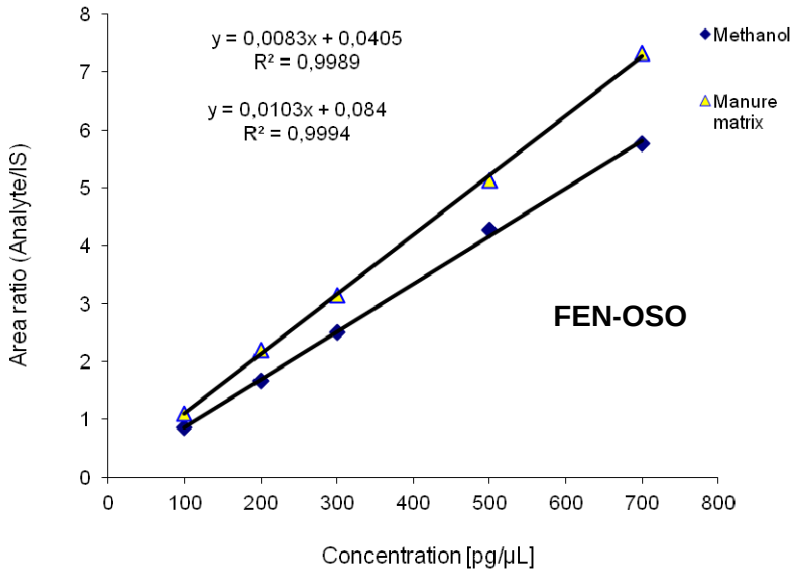


Figure 4.44: Continued.

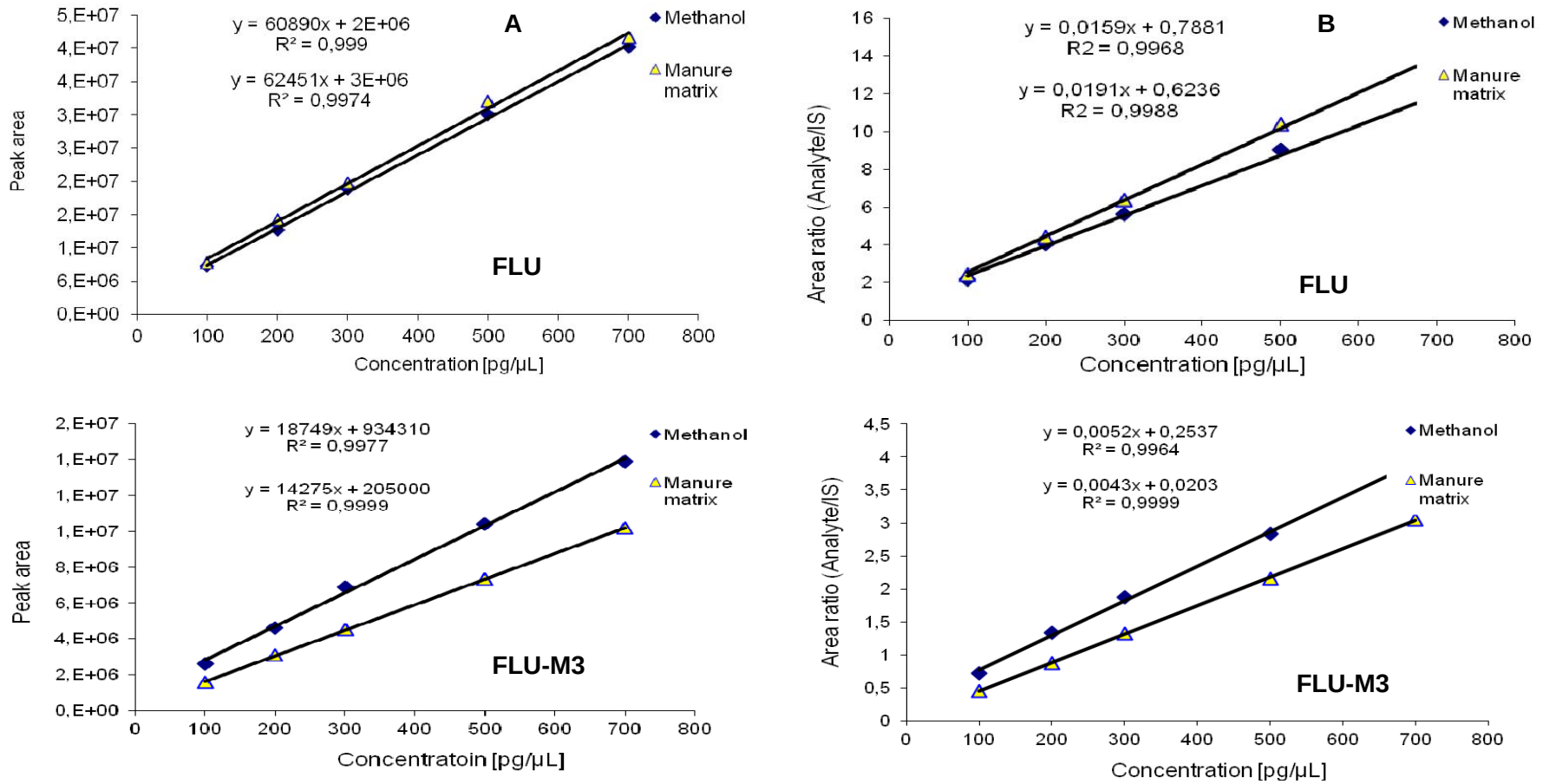


Figure 4.45: Calibration curves for flubendazole and corresponding metabolites A: External calibrations in methanol versus those recorded in manure matrix extracted after lyophilization with methanol, B: Internal calibration curves in the solvent vs. those recorded in manure matrix extracted after lyophilization with methanol

Table 4.26: Recoveries of fenbendazole and flubendazole with corresponding metabolites spiked to manured sand soil samples calculated by different calibration methods.

Analytes	Manured sand soil samples [100 µg/kg]		
	Absolute recovery (n = 3)		
	Matrix-matched	Internal calibration	External calibration
FEN	57 ± 4 (-24)*	47 ± 9	37 ± 6
FEN-SO	78 ± 5 (-17)*	78 ± 1	66 ± 5
FEN-OSO	77 ± 4 (-11)*	79 ± 7	67 ± 4
FLU	77 ± 5 (-15)*	88 ± 8	67 ± 5
FLU-M1	77 ± 6 (-5)*	80 ± 7	70 ± 6
FLU-M2	75 ± 4 (-17)*	73 ± 5	62 ± 1
FLU-M3	78 ± 10 (-27)*	72 ± 7	58 ± 3
FLU-M4	75 ± 6 (-13)*	84 ± 6	66 ± 1
FLU-M5	71 ± 6 (-15)*	69 ± 7	58 ± 3
FLU-M6	76 ± 5 (-33)*	61 ± 3	51 ± 3

* Matrix effects

The values in the practice are matrix effects for the target compounds calculated by comparing the peak areas of target compounds spiked to manure samples after the clean-up procedure with those of target compounds prepared in the solvent. These values showed a signal suppression of the target compounds, where internal standard showing also signal suppression (17 %) and has the ability to compensate the signal suppression for some of the target compounds.

4.7 Extraction efficiency and aging tests

In order to identify aging effects on the extractability of the FEN and FLU with corresponding metabolites in liquid pig manure, spiked liquid manure samples were incubated under anaerobic conditions up to 30 days. Test series were conducted at different temperatures. As typical for biotransformation tests, 20 °C was applied (OECD, 2000; 2002; Kreuzig, 2010; Kreuzig et al., 2010). Besides time-dependent losses of the extractability, decreasing recoveries can be also caused by transformation processes. In order to minimize biotransformation as far as possible by inhibiting the microbial activity during the 30-day incubation period, 4 °C was applied as well.

Table 4.27: Recoveries of fenbendazole and flubendazole with corresponding metabolites spiked to sand soil samples calculated by different calibration methods.

Analytes	Sand soil sample [100 µg/kg]		
	Absolute recovery (n = 3)		
	Matrix-matched	Internal calibration	External calibration
FEN	65 ± 14 (-7)*	54 ± 14	56 ± 14
FEN-SO	92 ± 8 (-2)*	85 ± 8	88 ± 8
FEN-OSO	83 ± 1 (+3)*	80 ± 1	83 ± 1
FLU	81 ± 1 (+2)*	79 ± 1	81 ± 1
FLU-M1	88 ± 1 (-2)*	81 ± 1	84 ± 1
FLU-M2	74 ± 10 (-12)*	61 ± 10	63 ± 9
FLU-M3	78 ± 9 (-1)*	72 ± 9	74 ± 15
FLU-M4	57 ± 15 (+5)*	54 ± 15	56 ± 7
FLU-M5	70 ± 7 (-12)*	57 ± 7	59 ± 2
FLU-M6	84 ± 2 (-1)*	79 ± 2	82 ± 2

* Matrix effects

The concentrations of target benzimidazole represented by the recoveries versus time during the aging tests are displayed in **Figure 4.46** and **4.47**. The obtained results at 4 ºC indicated that the recoveries of the target benzimidazoles were not affected by the time. Only the obtained recoveries of FLU-M5 were relatively low (50 to 60 %). At 20 °C, FLU-5 dropped even to 2-4 % after 30 days of incubation (**Figure 4.48**). In contrast, recoveries of FLU-M6 increased to 138 and 164 after 15 and 30 days of incubation even though at 4 ºC, there was not any change. Thus, the decrease of FLU-M5 may be caused by diffusion of this compound to less accessible sorption sites. Hence, the extractability may be reduced by the formation of physically entrapped or chemically bound residues. However, the simultaneous increase of FLU-M6 may indicate the transformation of one metabolite to the other.

An interesting behavior for FEN and metabolites were observed in the samples incubated at 20 ºC. Only 4 % of spiked FEN-SO was recovered after 30 days (**Figure 4.46**). At the same time an increase of FEN to 250 % of the initially applied amount was determined. The increase of the FEN recovery and the nearly complete disappearance of FEN-SO at 20 ºC may be attributed to the reduction of the oxidized metabolite to the parent compound. In contrast, FEN-OSO was recovered nearly unchanged during the 30-day incubation period. These results indicated that FEN, FLU and most of the metabolites are persistent in liquid pig manure. These results are consistent with results described by Kreuzig et al. (2007).

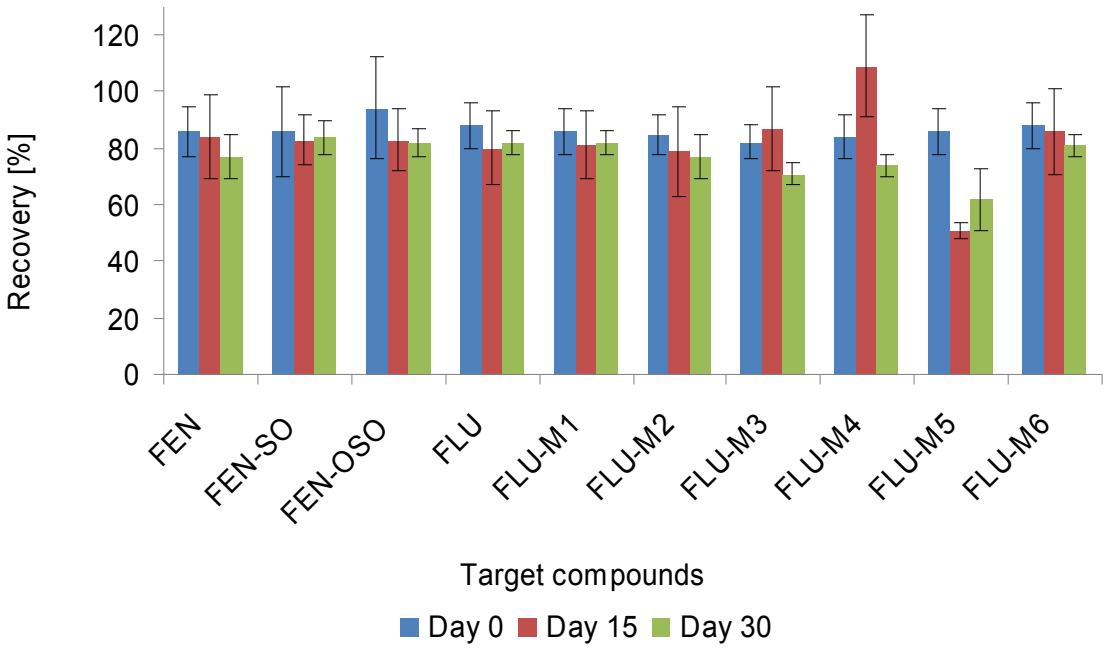


Figure 4.46: Recoveries of fenbendazole and flubendazole with corresponding metabolites in liquid pig manure incubated at 4 ºC up to 30 days after application.

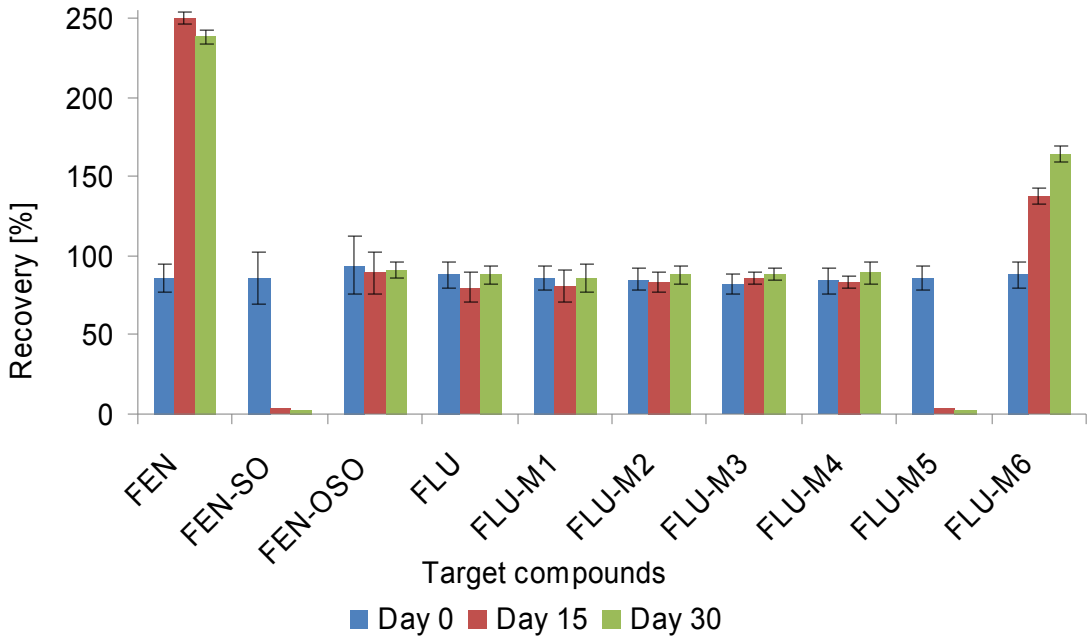


Figure 4.47: Recoveries of fenbendazole and flubendazole with corresponding metabolites in liquid pig manure incubated at 20 ºC up to 30 days after application.

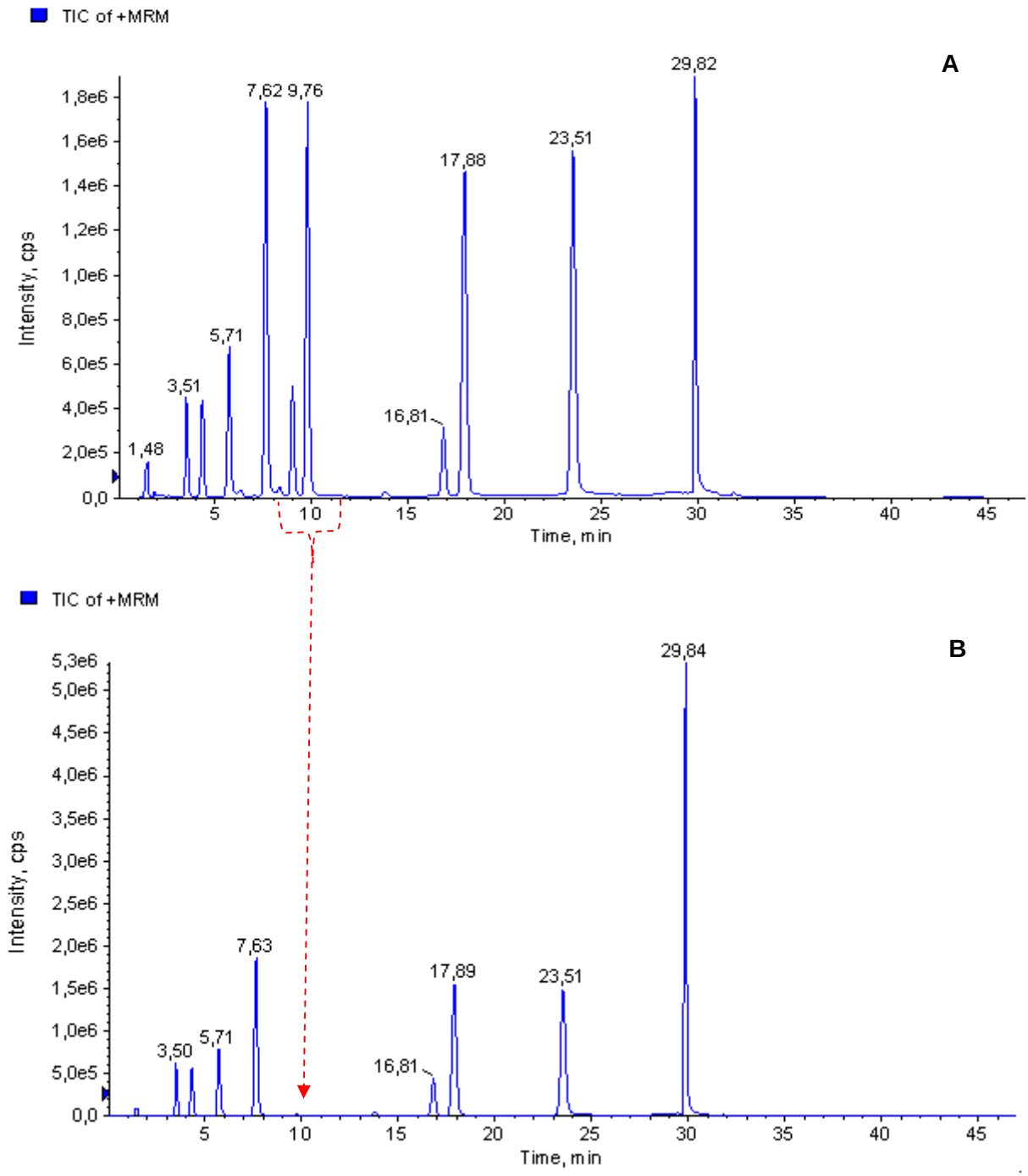


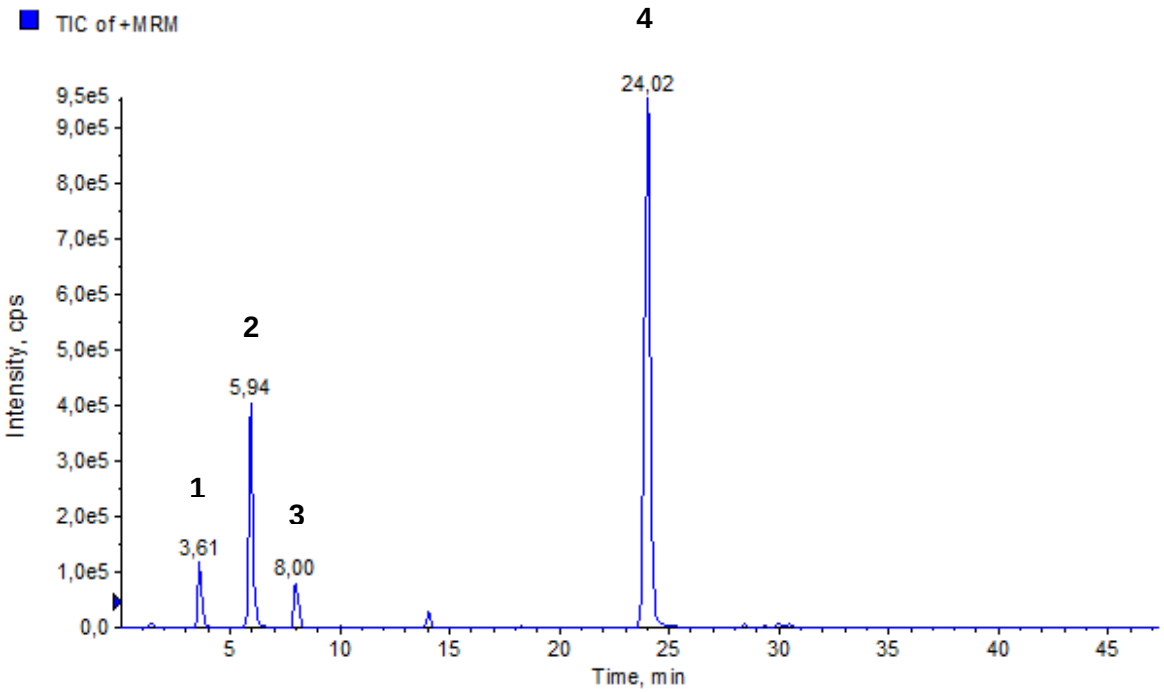
Figure 4.48: Total ion current chromatograms of spiked target compounds in liquid manure samples incubated at A: 4 ºC and B: 20 ºC for 30 days where FEN-SO and FLU-M5 were completely disappeared.

4.8 Analysis of real pig manure samples

The development of the new analytical method for the determination of FEN and FLU with corresponding metabolites inevitably started with fortification experiments in order to check for analytical handling and analytical quality assurance. For this purpose, reference manure was used to reduce the matrix heterogeneity of real manure samples and to avoid effects of other veterinary medicines and cleaning agents introduced into manures by agricultural practice. Therefore, feasibility and applicability of the method was finally checked by the analysis of real manure samples (PM1-PM7) from 7 pig fattening farms in the catchment area of the Chamber of Agriculture, Oldenburg, Germany, where only FLU was applied. Hence, neither FEN nor its metabolites were detected in any of the analyzed samples.
For the quantitation of the target compounds different techniques were compared. The use of external calibration to quantify the FLU and metabolites in a complex matrix such as liquid pig manure can have serious consequences, e.g., underestimation or overestimation due to unpredicted matrix effects as explained before. Although the matrix-matched calibration was a successful method in the fortification experiments in reference manure samples, this approach is impracticable in case of the real samples due to the highly variable compositions of real manures. Thus, the use of one manure matrix cannot display the composition of others ones that can be considerably different from one farm to another. This problem can be hardly solved when a single internal standard, i.e., the chloro-analogue of FLU, is applied. Therefore, multiple and single point standard addition were the most sophisticated techniques to perform correct quantitative analysis. During screening analysis for real manure sample, FLU, FLU-M1, FLU-M2 and FLU-M3 were detected in the real manure samples taken (**Figure 4.49**).

4.8.1 Multiple point standard addition

Besides the comparison of different quantitation methods, the manure samples PM1 and PM5 were directly ethyl acetate extracted and, alternatively, methanol extracted after lyophilization as described before, in order to check for the impact of different extract qualities. In contrast to the basic clean-up method using solid phase extraction, size exclusion chromatography was established as a further clean-up step. Then, the found concentrations were calculated by the multiple point standard addition technique. The results of both analytical approaches are listed in **Table 4.28.**



1: FLU-M3, 2: FLU-M2, 3: FLU-M1, 4: FLU

Figure 4.49: Total ion current chromatogram of the detected compounds in the real sample (PM1) extracted by ethyl acetate at pH 9.5.

Table 4.28: Calculated concentrations using multiple standard addition technique, where two extraction procedures used to extract real pig manure samples.

Analytes	Direct ethyl acetate extraction (pH 9.5)		Methanol extraction after lyophilization	
	PM1 (n = 2) [mg/kg]	PM5 (n = 2) [mg/kg]	PM1 (n = 2) [mg/kg]	PM5 (n = 2) [mg/kg]
FLU	0.32 ± 0.04	0.60 ± 0.08	0.28 ± 0.10	0.48 ± 0.04
FLU-M1	0.03 ± 0.00	0.02 ± 0.01	0.03 ± 0.01	0.01 ± 0.00
FLU-M2	0.10 ± 0.01	0.09 ± 0.00	0.09 ± 0.01	0.08 ± 0.002
FLU-M3	0.06 ± 0.04	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
FLU-M4	< MDL	< MDL	< MDL	< MDL
FLU-M5	< MDL	< MDL	< MDL	< MDL
FLU-M6	< MDL	< MDL	< MDL	< MDL

In the real manure samples PM1 and PM5, FLU and the metabolites, FLU-M1, FLU-M2 and FLU-M3 were identified and quantified. FLU occurred at average concentrations of 0.3 and 0.5 mg/kg accompanied by the metabolites at lower concentrations. Because FLU was not found to be substantially biodegradable in anaerobic biotransformation tests within a 100-day incubation periods (Kreuzig et al., 2007), here, the metabolic activity of the treated pigs was reflected. Thus, FLU was degraded via keton reduction forming FLU-M1 followed by its carbamate hydrolysis forming FLU-M3. Simultaneously, FLU directly underwent carbamate hydrolysis forming FLU-M2 (Van Leemput and Heykants, 1991). Further FLU metabolites could not be detected above method detection and quantitation limits.

For multiple standard addition technique, the real sample extracts were first analyzed for screening purposes using LC/MS/MS. The aliquots of the sample solutions were spiked at 100, 200, 300, 500 and 1000 pg/µL in specific volumes to record multiple standard addition curves. **Figure 4.50** shows the standard addition calibration curve as an example for this approach used to calculate FLU concentration PM1. Comparing both extraction methods showed similar extraction efficiencies. The deviation between the calculated concentrations was ≤ 13 in PM1 except for FLU-M3 (33 %). In PM5 the deviation was ≤ 20 % except in FLU-M1 (30%). This is probably due to the heterogeneity of the manure sample. Nevertheless, both approaches confirm that the developed method is feasible to determine the target benzimidazoles in real manure samples at high repeatability.

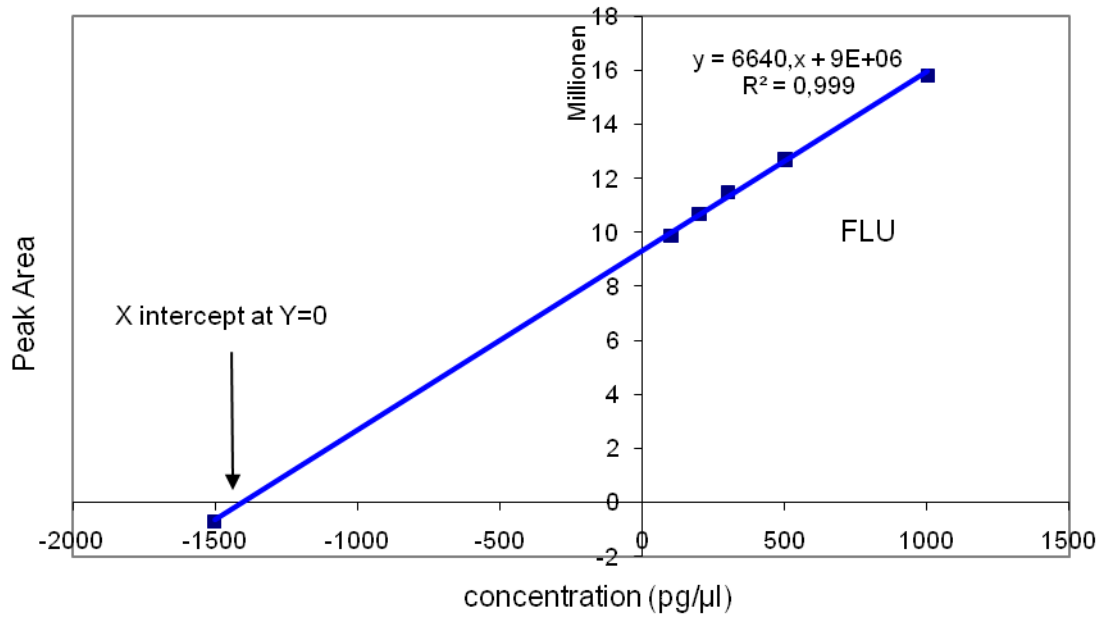


Figure 4.50: Multiple point standard addition plots used to determine the concentration of flubendazole where x intercept at y equal zero is the concentration of the target analyte in the real manure sample.

4.8.2 Single point standard addition

In these heterogeneous manure samples, losses during the clean-up procedure can be expected Therefore, it is impractical to correct the final results with fixed loss values based on the fortification experiments using reference manure samples different from the real manure samples. Therefore, the concentrations of detected compounds in the real samples extracted by ethyl acetate were additionally calculated using single point standard addition technique. The spiking step was after the extraction but before clean-up procedure where the matrix effect and the losses can be compensated. The results of this calibration approach are listed in **Table 4.29.**

Table 4.29: Concentrations of detected residues in real liquid pig manure samples after flubendazole administration to fattening pigs.

Pig manure sample	PM1 (n = 3) [mg/kg]	PM2 (n = 2) [mg/kg]	PM3 (n = 3) [mg/kg]	PM4 (n = 3) [mg/kg]	PM5 (n = 3) [mg/kg]	PM6 (n=3) [mg/kg]	PM7 (n = 3) [mg/kg]
ds [%]	11.1	3.6	1	5.4	9.6	2.7	2.8
TOC	61 ± 15	31 ± 0	17 ± 1	37 ± 1	36 ± 2	33 ± 1	26 ± 2
pH	7.5	7.3	7.4	7.6	7.4	8.0	7.7
FLU	0.14 ± 0.02	0.04 ± 0.003	0.16 ± 0.04	1.30 ± 0.3	0.6 ± 0.06	0.17 ± 0.04	0.10 ± 0.03
FLU-M1	0.01 ± 0.001	< LOQ	0.006 ± 0.001	0.01 ± 0.001	0.01 ± 0.001	0.01 ± 0.002	0.01 ± 0.001
FLU-M2	0.064 ± 0.01	0.01 ± 0.00	0.04 ± 0.004	0.06 ± 0.01	0.09 ± 0.01	0.06 ± 0.004	0.02 ± 0.003
FLU-M3	0.054 ± 0.004	< LOQ	0.03 ± 0.003	0.04 ± 0.001	0.08 ± 0.002	0.046 ± 0.003	<LOQ
FLU-M4	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL
FLU-M5	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL
FLU-M6	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL

FLU with FLU-M1, FLU-M2 and FLU-M3 were also detected in all other analyzed samples. Maximum concentrations were detected for FLU followed by FLU-M2, FLU-M3 and then FLU-M1, respectively, in all analyzed samples. These compounds were detected in PM4 and PM 5 with concentration higher than the other samples. The calculated concentrations in PM4 were 1.3 ± 0.03, 0.01 ± 0.01, 0.06 ± 0.01 and 0.04 ± 0.001 mg/kg fresh manure, respectively, for FLU, FLU-M1, FLU-M2 and FLU-M3, while in PM5 were 0.6 ± 0.06, 0.01 ± 0.001, 0.09 ± 0.01 and 0.08 ± 0.002 mg/kg fresh manure. FLU-M1 was not detected only in PM2, while FLU-M3 was not detected in PM2 and PM7. The results in **Table 4.29** show that there no correlation could be built depending on the concentration of detected compounds and the dry substance in each sample, e.g., compounds detected PM3 at nearly the same concentration like PM1 where the dry substance equal 1.1 % in the first and 11.1 % in the second.

Table 4.30 Calculated concentrations of flubendazole and corresponding metabolites using multiple and single point standard addition techniques.

Target compounds	Multiple point standard addition		Single point standard addition	
	PM1 (n = 2) [mg/kg]	PM5 (n = 2) [mg/kg]	PM1 (n = 3) [mg/kg]	PM5 (n = 3) [mg/kg]
FLU	0.32 ± 0.04	0.60 ± 0.08	0.14 ± 0.02	0.6 ± 0.06
FLU-M1	0.03 ± 0.00	0.02 ± 0.01	0.01 ± 0.001	0.01 ± 0.00
FLU-M2	0.10 ± 0.01	0.09 ± 0.00	0.064 ± 0.01	0.09 ± 0.01
FLU-M3	0.06 ± 0.04	0.05 ± 0.01	0.054 ± 0.004	0.08 ± 0.00
FLU-M4	< MDL	< MDL	< MDL	< MDL
FLU-M5	< MDL	< MDL	< MDL	< MDL
FLU-M6	< MDL	< MDL	< MDL	< MDL

Moreover, there were no relevant differences between the concentrations of the detected compounds calculated using single point and multiple point standard addition calibrations. Only the FLU-M1 concentration in PM5 calculated by single point standard addition was 50 % less than the value calculated by multiple point standard addition method. This may be attributed to the concentrations of the detected compounds were calculated after one month of sample collection using multiple standard addition technique and after 6 months using

single point standard addition technique. Another possible reason, this deviation may be attributed to the heterogeneity of this matrix. The same situation for FLU-M1 in PM1 was observed. Only the concentration of FLU decreased to be 44 % of its concentration calculated by multiple standard addition technique (**Table 4.30**).

4.8.3 Comparison of different quantitation methods

Different calibration methods were applied to calculate the concentrations of the compounds detected in PM1 and PM5 extracted by USE using methanol/ethyl acetate mixture (1:4, v/v, pH 9.5). In PM 1, the calculated concentrations using single point standard addition technique were 0.47 ± 0.1, 0.011 ± 0.001, 0.14 ± 0.02 and 0.06 ± 0.003 for FLU, FLU-M1, FLU-M2 and FLU-M3 mg/kg fresh manure, respectively but the concentration calculated by external calibration were 0.25 ± 0.02, 0.008 ± 0.0002, 0.09 ± 0.004 and 0.04 ± 0.001 mg/kg fresh manure for FLU, FLU-M1, FLU-M2 and FLU-M3, respectively. The deviations of the calculated concentrations by single point standard addition and external calibration indicated that the signals of the detected compounds were suppressed due to matrix effects. When the internal standard was added, increases of calculated concentrations up to 20 % were observed (**Table 4.31**). In general these results indicated that single point standard addition was an efficient method to calculate the concentrations of the detected compounds in this complex matrix.

Table 4.31: Calculated concentrations of flubendazole and corresponding metabolites using different quantitation methods. The samples were ultrasound-assisted extracted using ethyl acetate/methanol mixture (4:1 v/v, pH 9.5)

Calibration method	Single point standard addition		External calibration		Internal calibration	
Pig manure sample	PM1 (n = 4) [mg/kg ± SD]	PM5 (n = 4) [mg/kg ± SD]	PM1 (n = 4) [mg/kg ± SD]	PM5 (n = 4) [mg/kg± SD]	PM1 (n = 4) [mg/kg ± SD]	PM5 (n = 4) [mg/kg ± SD]
FLU*	0.47± 0.1	1.1 ± 0.31	0.25 ± 0.02	0.5 ± 0.002	0.3 ±0.016	0.66 ± 0.25
FLU-M1	0.011 ± 0.001	0.01 ± 0.002	0.008 ± 0.0002	0.005 ± 0.001	0.01±0.004	0.008 ± 0.001
FLU-M2	0.14 ± 0.02	0.11 ± 0.01	0.09 ± 0.004	0.075 ± 0.01	0.1±0.01	0.1 ± 0.005
FLU-M3	0.06 ± 0.003	0.09 ± 0.01	0.04 ± 0.001	0.06 ± 0.003	0.04±0.002	0.072 ± 0.004
FLU-M4	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL
FLU-M5	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL
FLU-M6	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL

*n =3

The losses during the clean-up procedure were considered during calculation of concentration using external and internal standards, where the loss were 27, 21, 26 and 20% of FLU, FLU-M1, FLU-M2 and FLU-M3, respectively.

4.9 Confirmation techniques

4.9.1 Concept of identification points

Different strategies were discussed to compensate the matrix effects which appeared as signal enhancement or suppression. Another important problem can occur due to the co-extractants especially for complex matrices such as manure and manured soil samples. Resulting problems are false positive and false negative findings. Besides the co-extractants, different reasons are responsible for the signal enhancement or signal suppression such as additives in the mobile phase of LC on chromatographic effects, e.g., increase or decrease of the flow rate or composition of the mobile phase. However, matrix effect is still only the main contributor about the false positive and false negative results, especially when isobaric compounds are present in the matrix under analysis. For this reason, several approaches were reported to confirm the identity of the organic pollutant in the samples under the study. The chromatographic behavior reflected by the retention times and absorption spectra may be considered as one confirmation method using HPLC/DAD so far both are significantly different. The Commission Decision 93/256/EEC thus states that "Methods based only on chromatographic analysis without the use of molecular spectrometric detection are not suitable for use as confirmatory methods". Another approach is the identification point (IP) concept in which the number of identification points depends on the technique used for the analysis of the compound under the study and its application, e.g., legal or illegal use (EC, 2002). The identification guideline for organic contaminants in animals and animal products according to the European Commission Decision 2002/657/EC states that at least three identification points (IPs) for legally applied compounds should be achieved. Thus 2 transitions each one act as 1.5 point and parent compound as 1 point are required for safely positive result confirmation using LC-MS/MS/MRM. In the present study using the 4000QTRAP LC-MS/MS system, 5 identification points for every compound were achieved: precursor ion (1 IP) and two transitions (3 IP) with retention time (IP). For highly qualified confirmation, all of the above criteria can combined with a comparison of not only the MRM transitions but also all the available transitions of each analyte in the real samples with those produced by standard solution under the same conditions. These were achieved using IDA facility in 4000QTRAP instrument, where MRM using two transitions was used as full scan and enhanced product ion scan as dependent scan. This approach was applied to check the obtained results from standard solution of the target compounds and spiked real samples and real sample. Examples of the IDA spectra produced using standard solution of mixture of benzimidazole compounds were compared to those produced using spiked and unspiked real sample in addition to the chromatographic behavior of these compounds are shown in

Figure 4.51 and **4.52**. Further mass spectra for other compounds are presented in **Appendix, Figure A4.**

Beside the specificity of the transitions selected in MRM method, qualified IPs should be achieved. For this, the ion ratio between selected transitions was calculated. All measured ion ratios must agree within specified tolerances. In LC/MS/MS, maximum permitted tolerances of ± 20 % for a relative ion intensity of > 50% to 50% for a relative ion intensity of ≤10 %. The both mentioned criteria play an important role in the safely confirmation procedure. The calculated ions ratios of the detected compounds in real manure samples were compared with those obtained in spiked real manure sample and reference standards. The confirmation was considered successful because the ratios deviations were lower than 20% (**Table 4.32).** Data for the other real samples are mentioned in **Appendix, Table A2 – A7.**

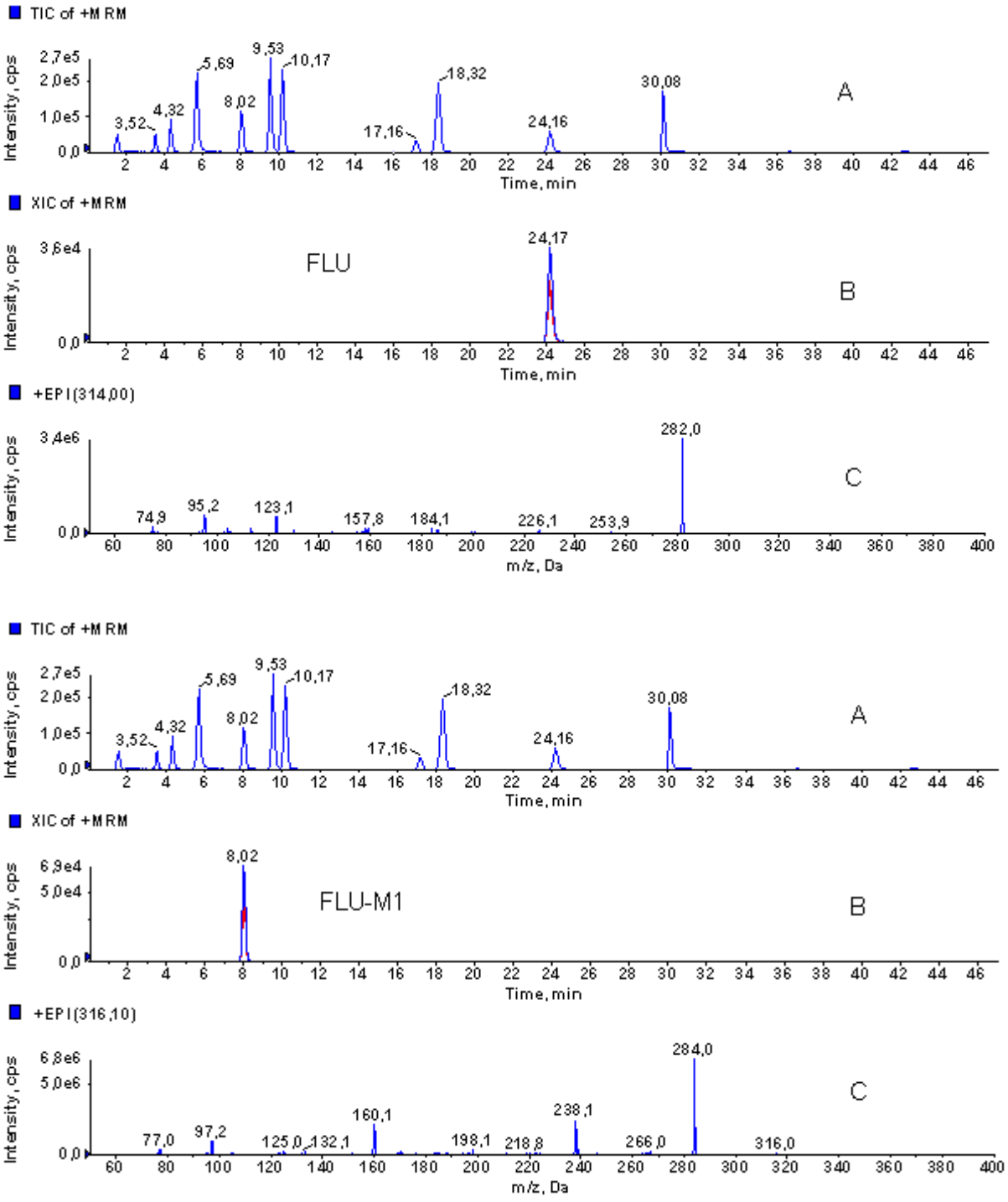


Figure 4.51: Total ion current chromatogram of A: flubendazole and corresponding metabolites (FLU-M1, FLU-M2 and FLU-M3 in methanol (200 pg/µL), B: extracted ion chromatograms and C: enhanced product ion spectra using information dependent aquisition (IDA).

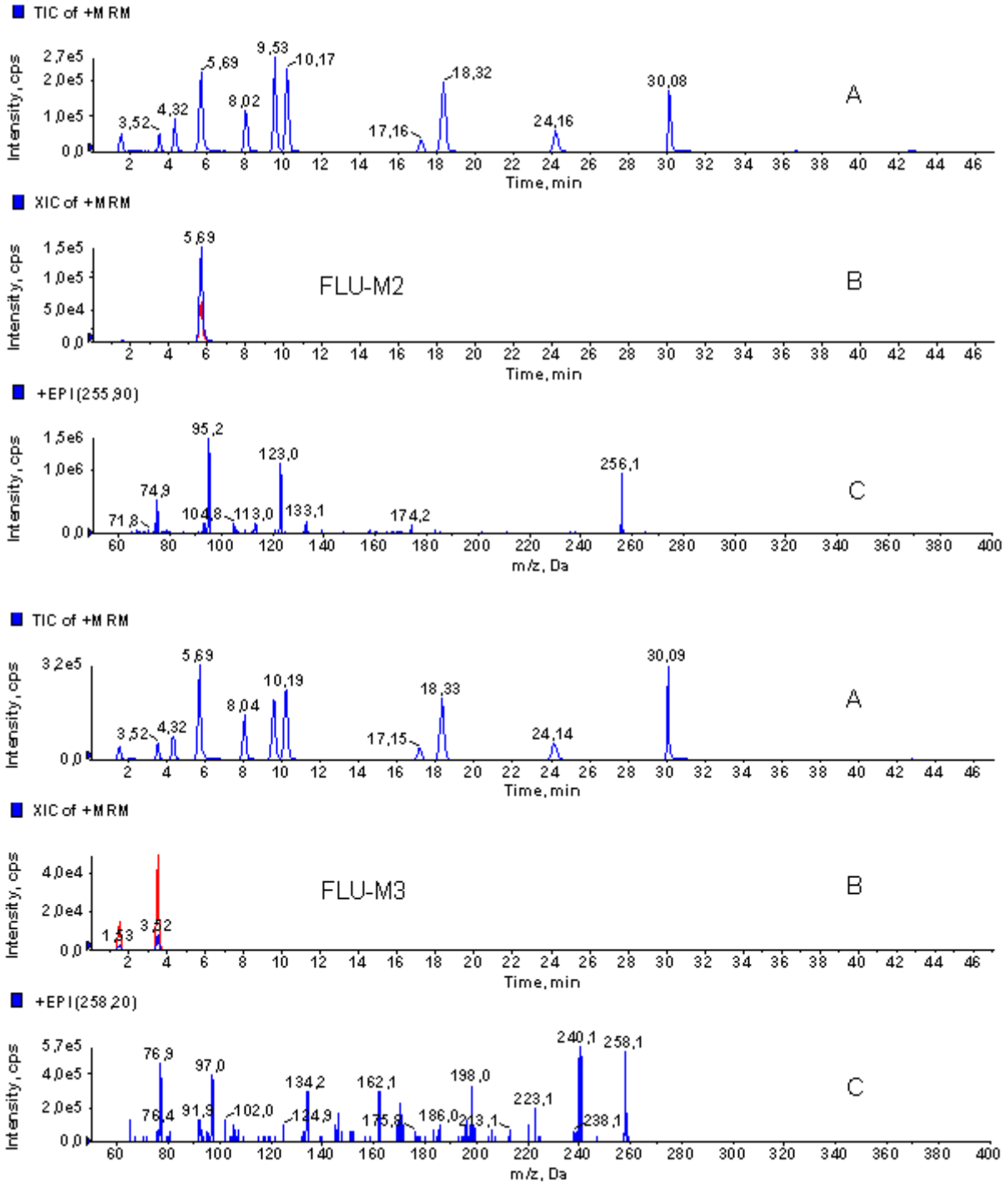


Figure 4.51: Continued.

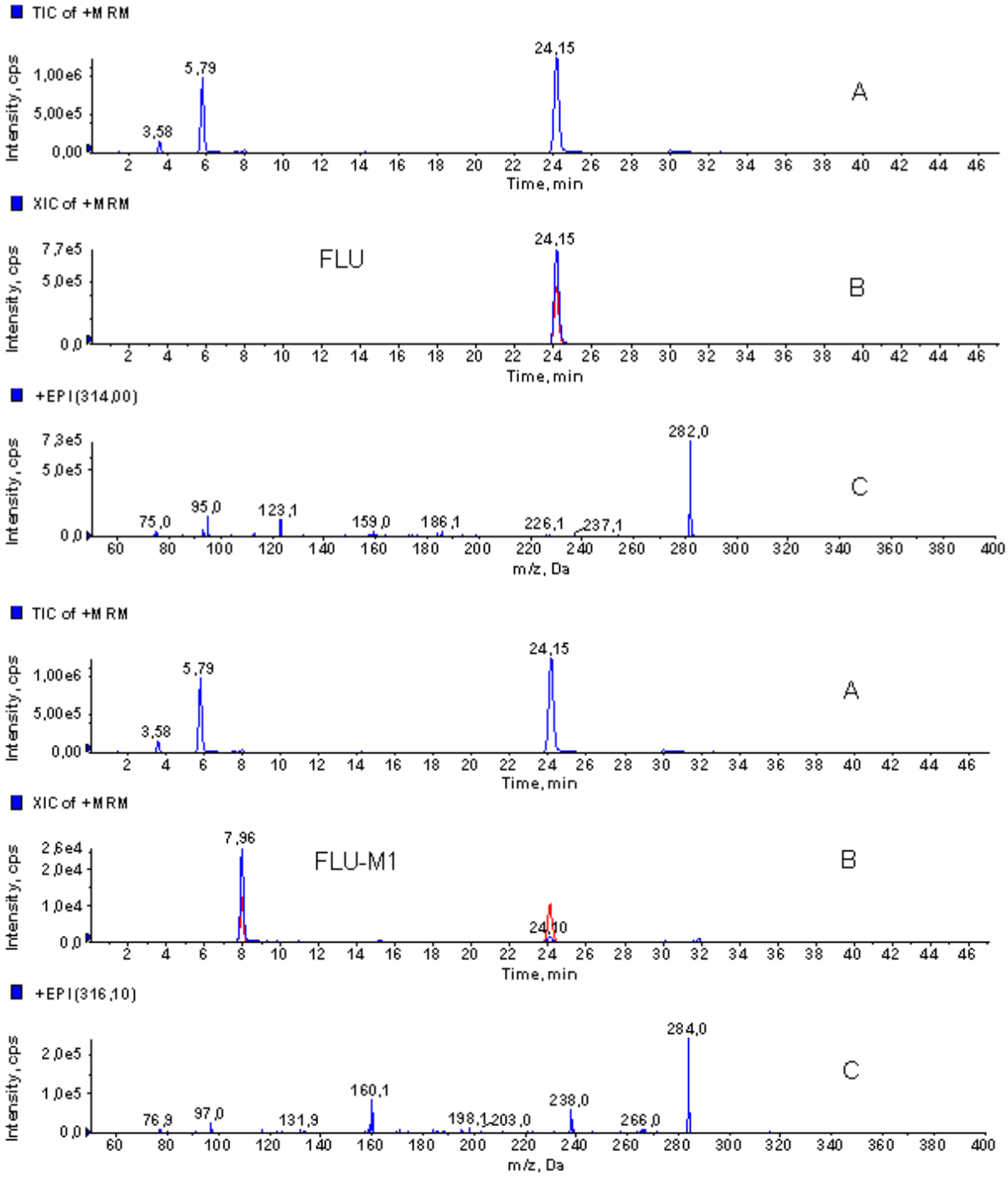


Figure 4.52: Total ion current chromatogram of A: FLU, FLU-M1, FLU-M2 and FLU-M3 detected in the real sample (PM1) ethyl acetate extracted, B: extracted ion chromatograms and C: enhanced product ion spectra.

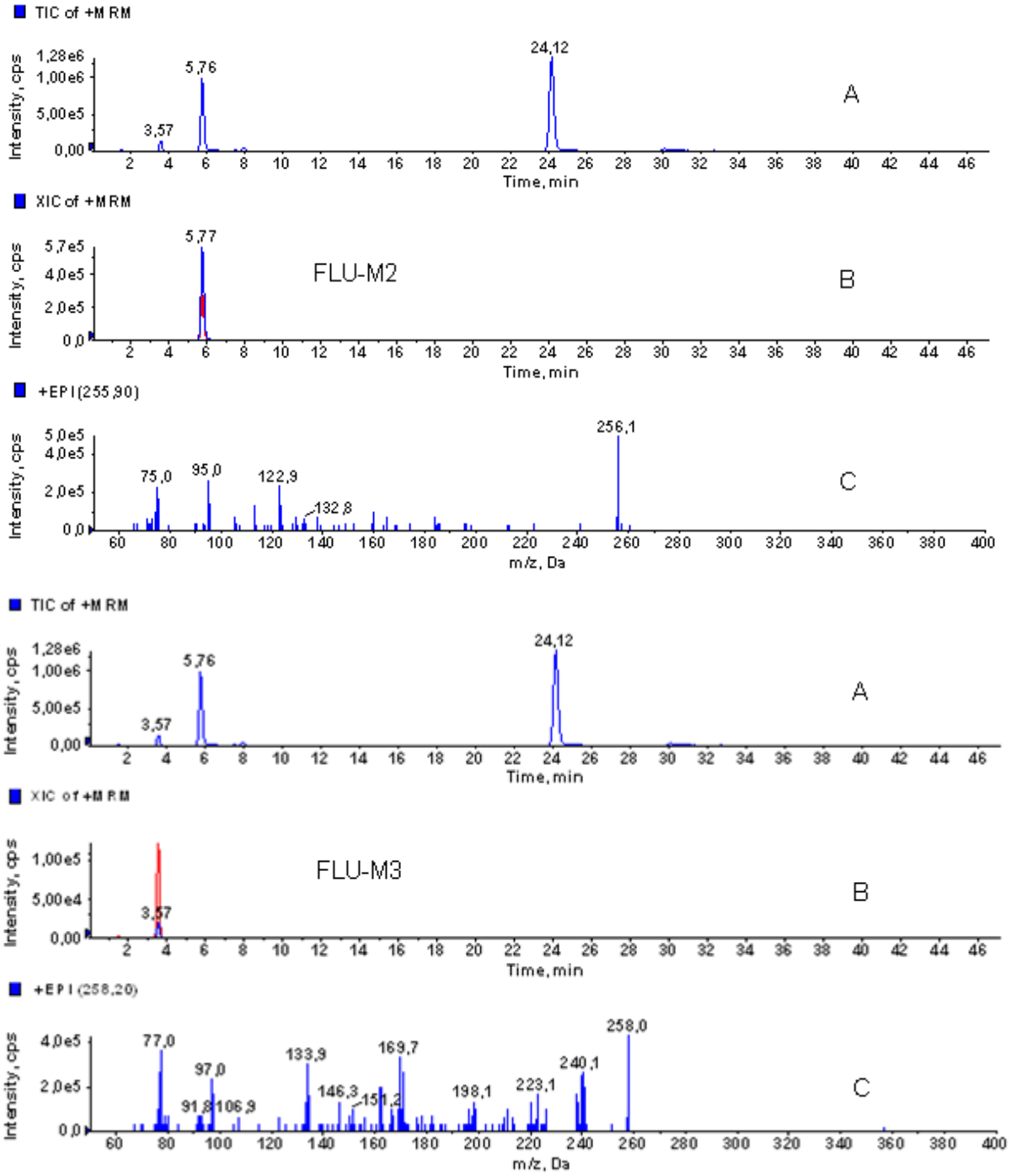


Figure 4.52: Continued.

Table 4.32: Confirmed results in PM1 according confirmation criteria in EU Commission Decision 2002/657/EC (EC, 2002).

Target compound	MRM-transitions (amu) (4 IP)	MRM ion ratios in standard solution (n=5)*	Permitted tolerance [%]	MRM ion ratios in real sample (PM1) (n=2)*	Deviation [%]	Result
FLU	314.20 -282.20 314.20 - 95.10	0.24	± 25	0.23	< 5	Confirmed
FLU-M1	316.20 -284.20 316.20 - 97.20	0.20	± 25	0.17	15	Confirmed
FLU-M2	256.00 - 95.00 256.00 -123.10	0.17	± 30	0.17	0	Confirmed
FLU-M3	258.10 -134.10 258.10 -240.30	0.30	± 25	0.29	< 4	Confirmed

*****RSD ≤ 8 %

4.9.2 Risk of false positive findings

The quantitation process of the target compounds using MRM method consisted of three steps: isolation of the precursor ions for each analyte, study of the product ions formed in the collision cell and selection of the transitions with highest intensity from different product ions for each analyte. Finally MRM method can be created. It is well known that MRM experiments are more specific and selective in comparison to SIM in single MS instruments, where the first mass analyzer acts as mass selector for the target compounds then fragmented in collision cell and specific two ions were selected to create MRM method. In such complex sample matrix like manure, numerous interfering compounds may have some ions at the same m/z like the target compounds under the study. The risk of false positive findings are still present if there is one or more interfering compound that has the same mass as precursor ions (isobaric molecules), especially if one or more of these isobaric molecules give the same fragment or fragments that are used in MRM method. This problem occurred during analysis of zero samples (blank) of manure methanol extracts after lyophilization. One compound was found with product ions of 123 and 95 m/z like FLU-M6. Its chromatographic behavior (RT 13.92 min) was completely different than FLU-M6 (RT. 16.98 min) as shown in **Figure 4.53**. It is apparent that these results required to use IDA method as detailed before to compare not only MRM ions but also all produced ions for the analytes in analyzed samples and standard solutions. **Figure 4.53** provides two approaches applied to distinguish between the unknown compound and FLU-M6. First is the retention time. The second is comparison of enhanced product ions spectra for the target compounds in the standard solutions and in the real samples extracts using IDA method. There are three shared ions between this interfering compound and FLU-M6 at m/z 123.0, 95.0 and 239.2 but the others are different. For examples, m/z 201.1, 184.1, 174.1, 157.1, 134.0, 113.0, 74.8 and 93.0 occur only in EPI of FLU-M3, while some ions occur only in the EPI of the interfering compound, e.g., 197.2, 183.1, 165.1, 152.1, 136.8, 103.0, 106.9 and 76.8 m/z. These results indicate that 4000 QTRAP system has the flexibility which allows improving the confirmatory data based on the IDA method.

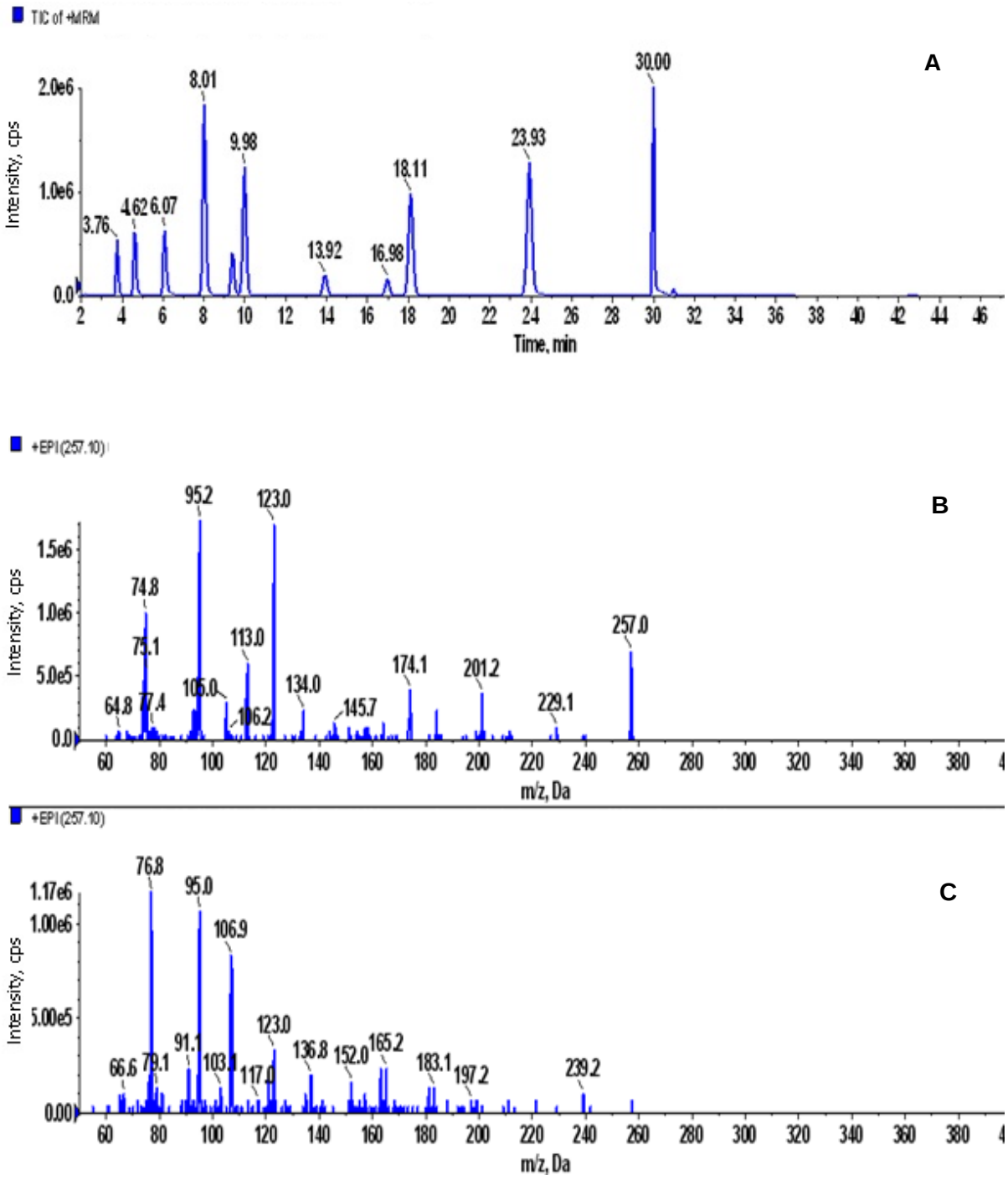


Figure 4.53: Total ion current chromatogram of A: fenbendazole and flubendazole with corresponding metabolites and an unknown compound (125 pg/µL in manure matrix), B and C: enhanced product ions spectra for FLU-M6 and the unknown compound in manure matrix and D: enhanced product ion spectrum of FLU-M6 in methanol for the standard solution.

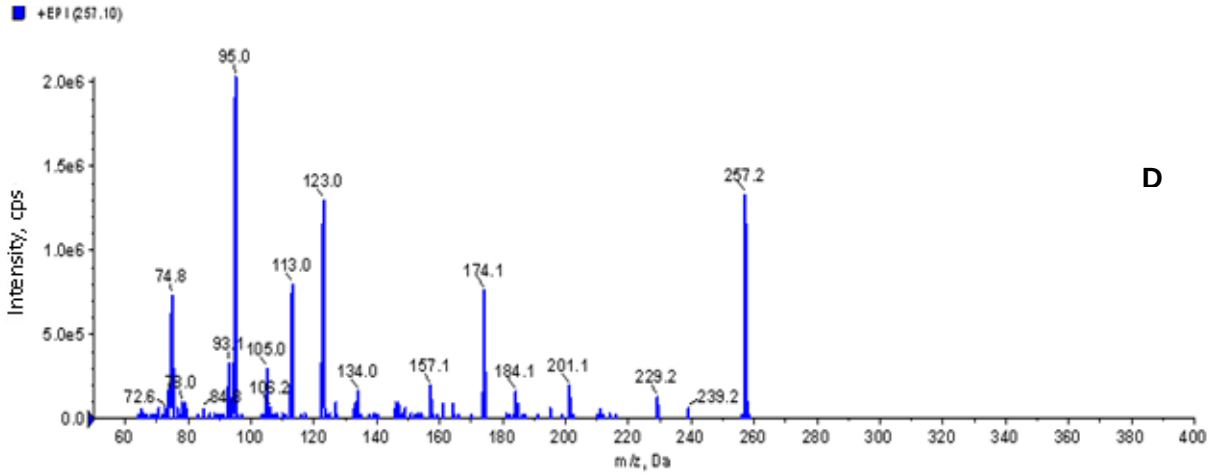


Figure 4.53: Continued.

5. Summary

Benzimidazole anthelmintics are one of the most important groups of veterinary medicines that are administered to production animals for the control of the endoparasites, i.e., gastrointestinal roundworms, lungworms and tapeworms. Over the last few years, these drugs have been frequently applied in Germany due to intensified livestock production. Among this group, fenbendazole and flubendazole are predominantly used in pig fattening and breeding farms. After drug administration, more than 50 % of these drugs are excreted together with corresponding metabolites via feces. Fenbendazole and flubendazole are not substantially degradable in liquid manures. Thus, the occurrence of these substances as well as corresponding metabolites in manured soils can not be excluded.

Even though benzimidazoles have been already analyzed in different environmental sample matrices, there does not exist any sophisticated analytical method for their determination in liquid pig manure and manured soil until today. Therefore, the objective of the present study focused on the development of an analytical method for the simultaneous determination of fenbendazole and flubendazole with corresponding metabolites in surface water, liquid pig manure, soil and manured soil.

In order to fulfil this goal, the analytical method development was performed in 4 steps. First, the extraction procedure was optimized using different solvent systems and different extraction techniques:

- Direct solvent extraction from liquid pig manure samples using ethyl acetate at pH 9.5 or lyophilization followed by methanol extraction at original pH.
- Ultrasound-assisted solvent extraction of lyophilized manure samples using a methanol/ethyl acetate mixture (1:4, v/v) at pH 9.5.
- Soil and manured soil samples were treated via direct solvent extraction or ultrasound-assisted extraction technique using different solvents and solvent mixtures.

Second, the raw extracts were cleaned up using size exclusion chromatography and solid phase extraction. Third, liquid chromatography coupled to tandem mass spectrometry with electrospray ionization in positive mode was applied to determine the target benzimidazoles at µg/kg concentrations. Fourth, different quantitation techniques, i.e., external, internal as well as single and multiple point standard addition, were applied in order to identify and compensate matrix effects. The results were finally confirmed by the identification point system.

This tiered experimental design was successfully applied for the identification and quantitation of the target benzimidazoles in spiked surface water, manure, soil and manured soil samples. As revealed by those fortification experiments for liquid manure samples method detection and quantitation limits ranged from 0.5 to 1 and from 1.6 to 3.1 µg/kg fresh manure. The obtained recovery ranged from 78 to 116 % , 76 to 106 % and 94 to 118 % with relative standard deviations of ≤ 19 % for the samples directly extracted using ethyl acetate at pH 9.5, lyophilization followed by methanol or ultrasound-assisted extraction of lyophilized samples using methanol/ethyl acetate mixture (1:4, v/v), respectively. Only the recovery of fenbendazole, i.e., 67 % with RSD = 4 % at 50 µg/kg spiking level, was marginally below the acceptable recovery range.

For soil and manured soil samples, method detection limits ranged from 0.3 to 1.2 µg/kg and from 0.7 to 2.5 µg/kg, while method quantitation limits ranged from 0.8 to 3.7 µg/kg and from 2.4 to 7.9 µg/kg, respectively. The overall recovery rates ranged from 70 to 119 % and relative standard deviations were less than 21 %. Only at 4 µg/kg, recovery rates were 56 and 120 % for fenbendazole and one flubendazole metabolite. A comparison of direct solvent extraction and ultrasound-assisted extraction techniques revealed that both methods are suitable to reach the acceptaple recovery range of > 70 %.

The obtained results have already confirmed the high precision and accuracy of the developed analytical method at low µg/kg concentrations of the target benzimidazoles. In addition to these fortification tests, carried out under best-case conditions, anaerobic biotransformation tests were conducted to study the aging effect on the extractability of the spiked target compounds from liquid manure samples after a 30-day incubation period. At 20 ºC, only 2-4 % of the sulfoxide metabolite of fenbendazole were recovered while the fenbendazole concentration increased up to 250 % of the initially applied amount indicating the regeneration of the parent compound from its primary metabolite. Similarities were also found for 2 flubendazole metabolites. The recovery rates of the other compounds were ≥ 86 %.Furthermore, the obtained results at 4 ºC indicated that the extraction efficiency of the target benzimidazoles from manure samples was not affected by aging processes.

From different pig fattening farms in the catchment area of the Chamber of Agriculture, Oldenburg, Germany, real pig manure samples were taken and analyzed in order to check the applicability of the developed method. Flubendazole was administered as a food additive at 5 mg/kg pig body weight for 5 consecutive days. Flubendazole occurred at concentrations of 0.1 and 1.3 mg/kg fresh manure accompanied by the metabolites at lower concentrations from 0.006 to 0.09 mg/kg fresh manure.

External, internal and standard addition calibrations were applied to calculate the concentrations in two real samples extracted by ultrasound-assisted extraction with methanol/ethyl acetate mixture (1:4, v/v, pH 9.5). The deviations between the obtained

results by the single point standard addition and the external calibration indicated that the signals of the detected compounds were suppressed due to matrix effects. When the internal standard was added, an increase of concentrations up to 20 % was observed but still less than those obtained by the single point standard addition method, reflecting incomplete compensation of the matrix effects. In general, these results indicated that the standard addition techniques were the most suitable methods to perform correct quantitative analysis.

The positive results were confirmed in accordance with the European Commission Decision 2002/657/EC for confirmation and identification of organic pollutants. Five identification points (IP) for every compound were achieved: Precursor ion (1 IP) and two mass transitions (3 IP) with retention time (1 IP). For more qualified confirmation, these indentification points were additionally combined with the comparison of the entity of detected ions of each analyte in the real samples with those produced by standard solution under the same conditions. For this purpose, the information dependent acquisition was performed using multiple reaction monitoring for two mass transitions as full scan mode while enhanced product ion scan was used as dependent scan. Because 5 identification points were obtained and the ion ratio deviations were lower than 20 %, safely confirmations of positive finding were successfully achieved meeting the current requirements of analytical quality assurance.

This new analytical method facilitates the determination of fenbendazole and flubendazole with corresponding metabolites in liquid manures and manured soils. Hence, screening and monitoring studies at animal husbandry farms can be performed now in order to check for the relevance of the manure application as an possible entry route of benzimidazole anthelmintics into agricultural soils. Furthermore, laboratory tests on anaerobic biotransformation in liquid manures and aerobic biotransformation in manured soils can be advanced now by taking the corresponding metabolites into special account. Finally, the applied experimental design can be used for further method development for the determination of other veterinary medicines in liquid manures and manured soils. In any case, this new analytical method can contribute to improve studies on the prospective evaluation of veterinary medicines within the regulatory procedures required by the authorities.

6. References

Abahussain, E.A., Ball, D.E., Matowe, W.C. (2006): Practice and opinion towards disposal of unused medication in Kuwait. Medical Principles and Practice. International Journal of the Kuwait University Health Science Center, 15 352-357.

AB Sciex (2009): Training program-Basic Operator Training course, http://www.absciex.com/Documents/Downloads/Literature/mass-spectrometry-cms_039796.pdf., retrieved March 17, 2012

Achtenhagen, J., Kreuzig, R. (2011): Laboratory tests on the impact of superabsorbent polymers on transformation and sorption of xenobiotics in soil taking ^{14}C-imazalil as an example. Science of the Total Environment, 409, 5454-5458.

Adolfsson-Erici, M., Pettersson, M., Parkkonen, J., Sturve, J. (2002): Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden. Chemosphere, 46, 1485-1489.

Agilent ZORBAX Column Selection Guide for HPLC. http://www.chromtech.com/Catalog/ProductLine/brochures/Zorbax_Guide.pdf., retrieved March 17, 2012.

Aguilera-Luiz, M.M., Martínez Vidal, J.L., Romero-González, R., Garrido Frenich, A. (2012): Multiclass method for fast determination of veterinary drug residues in baby food by ultra-high-performance liquid chromatography-tandem mass spectrometry. Food Chemistry, 132, 2171-2180.

Ahrer, W., Scherwenk, E., Buchberger, W. (2001): Determination of drug residues in water by the combination of liquid chromatography or capillary electrophoresis with electrospray mass spectrometry. Journal of Chromatography A, 910, 69-78.

Antignac, J.-P., de Wasch, K., Monteau, F., De Brabander, H., Andre, F., Le Bizec, B. (2005): The ion suppression phenomenon in liquid chromatography–mass spectrometry and its consequences in the field of residue analysis. Analytica Chimica Acta, 529, 129-136.

Andersson, A.M., Skakkebaek, N.E. (1999): Exposure to exogenous estrogens in food: possible impact on human development and health. European Journal of Endocrinology, 140, 477-485.

Aydin, M.E., Ozcan, S., Tor, A. (2007): Ultrasonic solvent extraction of persistent organic pollutants from airborne particles. CLEAN – Soil, Air, Water, 35, 660-668.

A. Żwir-Ferenc, M.B. (2006): Solid phase extraction technique – trends, opportunities and applications. Polish Journal of Environmental Studies, 15, 677-690.

Babic, S., Petrovic´, M., Kasˇtelan-Macan, M. (1998): Ultrasonic solvent extraction of pesticides from soil. Journal of Chromatography A, 823, 3-9.

Balizs, G. (1999): Determination of benzimidazole residues using liquid chromatography and tandem mass spectrometry. Journal of Chromatography B: Biomedical Sciences and Applications, 727, 167-177.

Barker, S.A., Hsieh, L.C., McDowell, T.R., Short, C.R. (1987): Qualitative and quantitative analysis of the anthelmintic fenbendazole and its metabolites in biological matrices by direct exposure probe mass spectrometry. Biomedical and Environmental Mass Spectrometry, 14, 161-165.

Barron, L., Tobin, J., Paull, B. (2008): Multi-residue determination of pharmaceuticals in sludge and sludge enriched soils using pressurized liquid extraction, solid phase extraction and liquid chromatography with tandem mass spectrometry. Journal of Environmental Monitoring, 10, 353-361.

Batt, A.L., Aga, D.S. (2005): Simultaneous analysis of multiple classes of antibiotics by ion trap LC/MS/MS for assessing surface water and groundwater contamination. Analytical Chemistry, 77, 2940-2947.

Bauer, C., Gerwert, S. (2002): Characteristics of a flubendazole resistant isolate of *Oesophagostomum dentatum* from Germany. Veterinary Parasitology, 103, 89-97.

Belfroid, A.C., Van der Horst, A., Vethaak, A.D., Schäfer, A.J., Rijs, G.B., Wegener, J., Cofino, W.P. (1999): Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in the Netherlands. Science of the Total Environment, 225, 101-108.

Blanchflower, W.J.C., Andrew, Kennedy, D.G. (1994): Determination of fenbendazole and oxfendazole in liver and muscle using liquid chromatography-mass spectrometry. Analyst, 119, 1325-1328.

Boqué, B.R., Maroto, A., Riu, J., Rius, F.X. (2002): Validation of analytical methods. Grasas y Aceites, 53, 128-143.

Borgmann, U., Bennie, D.T., Ball, A.L., Palabrica, V. (2007): Effect of a mixture of seven pharmaceuticals on *Hyalella azteca* over multiple generations. Chemosphere, 66, 1278-1283.

Botsoglou, N.A., Fletouris, D.J., Psomas, I.E., Mantis, A.I. (1997): Retention profile of fenbendazole and its sulphoxide , sulphone , and hydroxylated metabolites in ion-pair liquid chromatography. Journal of Liquid Chromatography and Related Technologies, 20, 1175-1184.

Botsoglou, N.A., Fletouris, J.D. (2001): Drug residues in foods: Pharmacology, safety, and analysis. Marcel Dekker AG, Hutgasse 4, Postfach 812, CH-4001 Basel, Switzerland.

Boxall, A.B.A., Kolpin, D.W., Halling-Sørensen, B., Tolls, J. (2003): Are veterinary medicines causing environmental risks? Environmental Science and Technology, 36, 286-294.

Boyd, G.R., Palmeri, J.M., Zhang, S., Grimm, D.A. (2004): Pharmaceuticals and personal care products (PPCPs) and endocrine disrupting chemicals (EDCs) in stormwater canals and Bayou St. John in New Orleans, Louisiana, USA. Science of the Total Environment, 333, 137-148.

Brandon, D.L., Bates, A.H., Binder, R.G., Montague, W.C., Whitehand, L.C., Barker, S.A. (2002): Analysis of fenbendazole residues in bovine milk by ELISA. Journal of Agricultural and Food Chemistry, 50, 5791-5796.

Brandon, D.L., Binder, R.G., Bates, A.H., Montague, W.C. (1994): Monoclonal antibody for multiresidue ELISA of benzimidazole anthelmintics in liver. Journal of Agricultural and Food Chemistry, 42, 1588-1594.

Bruins, A.P. (1994): Atmospheric pressure ionization mass spectrometry. Trends in Analytical Chemistry, 13, 81-90.

Burton, C.H., Turner, C. (2003): Manure management: Treatment strategies for sustainable agriculture. Silsoe Research Institute, Wrest Park, Bedford, UK, ISBN 0953128261, 31-34.

Buser, H.-R., Poiger, T., Müller, M.D. (1999): Occurrence and environmental behavior of the chiral pharmaceutical drug Ibuprofen in surface waters and in wastewater. Environmental Science and Technology, 2529-2535.

Buser, H.-R., Müller, M.D. (1998a): Occurrence of the pharmaceutical drug clofibric acid and the herbicide mecoprop in various Swiss lakes and in the North Sea. Environmental Science and Technology, 32, 188-192.

Buser, H.-R., Pioger, T., Müller, M.D. (1998b): Occurrence and fate of the pharmaceutical drug diclofenac in surface waters: Rapid photodegradation in a lake. Environmental Science and Technology, 32, 3449-3456.

Caprioli, G., Cristalli, G., Galarini, R., Giacobbe, D., Ricciutelli, M., Vittori, S., Zuo, Y., Sagratini, G. (2010): Comparison of two different isolation methods of benzimidazoles and their metabolites in the bovine liver by solid-phase extraction and liquid chromatography-diode array detection. Journal of Chromatography A, 1217, 1779-1785.

Careri, M., Mangia, A. (2006): Validation and qualification: The fitness for purpose of mass spectrometry-based analytical methods and analytical systems. Analytical and Bioanalytical Chemistry, 386, 38-45.

Castiglioni, S., Bagnati, R., Fanelli, R., Pomati, F., Calamari, D., Zuccato, E. (2006): Removal of pharmaceuticals in sewage treatment plants in Italy. Environmental Science and Technology, 40, 357-363.

Cha, J.M., Yang, S., Carlson, K.H. (2006): Trace determination of beta-lactam antibiotics in surface water and urban wastewater using liquid chromatography combined with electrospray tandem mass spectrometry. Journal of Chromatography A, 1115, 46-57.

Christian, T., Schneider, R.J., Färber, H.A., Skutlarek, D., Meyer, M.T., Goldbach, H.E. (2003): Determination of antibiotic residues in manure, soil, and surface waters. Acta Hydrochimica et Hydrobiologica, 31, 36-44.

Cvilink, V., Szotakova, B., Vokral, I., Bartıkova, H., Lamka, J., Skalova, L. (2009): Liquid chromatography/mass spectrometric identification of benzimidazole anthelminthics metabolites formed ex vivo by Dicrocoelium dendriticum. Rapid Communications in Mass Spectrometry, 23 , 2679-2684.

Danaher, M., De Ruyck, H., Crooks, S.R.H., Dowling, G., O'Keeffe, M. (2007): Review of methodology for the determination of benzimidazole residues in biological matrices. Journal of Chromatography B, 845, 1-37.

Danaher, M. (2003): Development and optimisation of a method for the extraction of benzimidazoles from animal liver using supercritical carbon dioxide. Analytica Chimica Acta, 483, 313-324.

Daughton, C.G., Ternes, T.A. (1999): Pharmaceuticals and personal care products in the environment: agents of subtle change? Environmental Health Perspectives, 107, 907-938.

Dayan, A.D. (2007): Risk assessment of triclosan [Irgasan] in human breast milk. Food and Chemical Toxicology an international journal published for the British Industrial Biological Research Association, 45, 125-129.

Díaz-Cruz, M.S., Barceló, D. (2006): Determination of antimicrobial residues and metabolites in the aquatic environment by liquid chromatography tandem mass spectrometry. Analytical and Bioanalytical Chemistry, 386, 973-985.

De Oliveira, M.F., Stradiotto, N.R. (2002): Voltammetric determination of fenbendazole in veterinarian formulations. Journal of Pharmaceutical and Biomedical Analysis, 30, 279-284.

De Ruyck, H., Daeseleire, E., De Ridder, H., Van Renterghem, R. (2002): Development and validation of a liquid chromatographic-electrospray tandem mass spectrometric multiresidue method for anthelmintics in milk. Journal of Chromatography A, 976, 181-194.

De Ruyck, H., Daeseleire, E., Grijspeerdt, K., De Ridder, H., Van Renterghem, R., Huyghebaert, G. (2001): Determination of flubendazole and its metabolites in eggs and poultry muscle with liquid chromatography-tandem mass spectrometry. Journal of Agricultural and Food Chemistry, 49, 610-617.

De Ruyck, H., Van Renterghem, R., De Ridder, H., De Brabander, D. (2000): Determination of anthelmintic residues in milk by high performance liquid chromatography. Food Control, 11, 165-173.

Dec Bronsvoort, B.M., Makepeace, B.L., Renz, A., Tanya, V.N., Fleckenstein, L., Ekale, D., Trees, A.J. (2008): UMF-078: A modified flubendazole with potent macrofilaricidal activity against *Onchocerca ochengi* in African cattle. Parasites and Vectors, 1, 18.

DellaGreca, M., Brigante, M., Isidori, M., Nardelli, A., Previtera, L., Rubino, M., Temussi, F. (2003): Phototransformation and ecotoxicity of the drug naproxen-Na. Environmental Chemistry Letters, 1, 237-241.

Delle Site, A. (2001): Factors affecting sorption of organic compounds in natural sorbent/water systems and sorption coefficients for selected pollutants. Journal of Physical and Chemical Reference Data, 30, 194-227.

Diiwel, D. (1977): Fenbendazole. II. Biological properties and activity. Journal of Pesticide Science, 8, 550-555.

Ding, C., He, J. (2010): Effect of antibiotics in the environment on microbial populations. Applied Microbiology and Biotechnology, 87, 925-941.

Dowling, G., Cantwell, H., Okeeffe, M., Smyth, M. (2005): Multi-residue method for the determination of benzimidazoles in bovine liver. Analytica Chimica Acta, 529, 285-292.

Drillia, P., Stamatelatou, K., Lyberatos, G. (2005): Fate and mobility of pharmaceuticals in solid matrices. Chemosphere, 60, 1034-1044.

Dusi, G., Gamba, V., Faggionato, E. (2005): Rapid determination of the antiparasitic drugs flubendazole and febantel in feeds by HPLC with ultraviolet detection. Journal of Pharmaceutical and Biomedical Analysis, 38, 375-379.

Edward, B., Henion, J. (1998): Atmospheric pressure ionization LC / MS / MS techniques for drug disposition. Journal of Pharmaceutical Scinces, 87, 395-402.

EC, European Commission Decision 2002/657/EC Implementing Council Directive 96/23/EC, concerning the performance of analytical methods and the interpretation of results. http://faolex.fao.org/docs/pdf/eur49615.pdf. retrieved, March 17, 2012.

European Medicines Agency. (2006): EMEA/CVMP/33128/2006-Final, flubendazole summary report 4. http://www.emea.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-_Report/2009/11/WC500014292.pdf, retrieved March 15, 2012.

European Medicines Agency. (2004a): EMEA/MRL/866/03-Final, fenbendazole summary report 4. http://www.ema.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-_Report/2010/07/WC500094856.pdf, retrieved March 15, 2012.

European Medicines Agency. (2004b): EMEA/MRL/888/03-Final, fenbendazole sulfoxide summary report 4. http://www.emea.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-_Report/2009/11/WC500015330.pdf, retrieved March 15, 2012.

European Medicines Agency. (2004c): EMEA/MRL/867/03-Final, febantel summary report 4. http://www.emea.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-_Report/2009/11/WC500014242.pdf, retrieved March 15, 2012.

Farré, M.L., Ferrer, I., Ginebreda, A., Figueras, M., Olivella, L., Tirapu, L., Vilanova, M., Barceló, D. (2001): Determination of drugs in surface water and wastewater samples by liquid chromatography-mass spectrometry: Methods and preliminary results including toxicity studies with *Vibrio fischeri*. Journal of Chromatography A, 938, 187-197.

Fent, K., Weston, A.A., Caminada, D. (2006): Ecotoxicology of human pharmaceuticals. Aquatic Toxicology, 76, 122-159.

Fletouris, D.J., Botsoglou, N.A., Psomas, I.E., Mantis, A.I., Regulation, C. (1996): Trace multiresidue analysis of fenbendazole and its sulfoxide, sulfone and p-hydroxylated metabolites in milk by liquid chromatography. Journal of Agricultural and Food Chemistry, 44, 3882-3886.

Fletouris, D.J., Botsoglou, N.A., Psomas, I.E., Mantis, A.I. (1994): Rapid ion pair liquid chromatographic method for detremination of fenbendazole in cows' milk. Analyst, 119, 2801-2804.

Frayha, G.J., Smyth, J.D., Gobert, J.G., Savel, J. (1997): The mechanisms of action of antiprotozoal anthelmintic drugs in man. General Pharmacology, 28, 273-299.

Garrido Frenich, A., Martínez Vidal, J.L., Fernández Moreno, J.L., Romero-González, R. (2009): Compensation for matrix effects in gas chromatography-tandem mass spectrometry using a single point standard addition. Journal of chromatography. A, 1216, 4798-4808.

Gokbulut, C., Akar, F., McKellar, Q.A. (2006): Plasma disposition and faecal excretion of oxfendazole, fenbendazole, and albendazole following oral administration to donkeys. Veterinary Journal, 172, 166-172.

Golet, E.M., Alder, A.C., Hartmann, A., Ternes, T.A., Giger, W. (2001): Trace determination of fluoroquinolone antibacterial agents in urban wastewater by solid-phase extraction and liquid chromatography with fluorescence detection. Analytical Chemistry, 73, 3632-3638.

Goossens, H., Ferech, M., Vander Stichele, R., Elseviers, M. (2005): Outpatient antibiotic use in Europe and association with resistance: A cross-national database study. Lancet, 365, 579-587.

Gosetti, F., Mazzucco, E., Zampieri, D., Gennaro, M.C. (2010): Signal suppression/enhancement in high-performance liquid chromatography tandem mass spectrometry. Journal of Chromatography. A, 1217, 3929-3937.

Gottschall, D.W., Theodorides, V.J., Wang, R. (1990): The metabolism of benzimidazole anthelmintics. Parasitology Today, 6, 115-124.

Griffiths, W.J., Jonsson, A.P., Liu, S., Rai, D.K., Wang, Y. (2001): Electrospray and tandem mass spectrometry in biochemistry. Biochemical Journal, 355, 545-561.

Gros, M., Petrović, M., Barceló, D. (2006): Development of a multi-residue analytical methodology based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) for screening and trace level determination of pharmaceuticals in surface and wastewaters. Talanta, 70, 678-690.

Hager, J.W. (2002): A new linear ion trap mass spectrometer. Rapid Communications in Mass Spectrometry, 16, 512-526.

Haller, M., Muller, S., Mcardell, C., Alder, A., Suter, M. (2002): Quantification of veterinary antibiotics (sulfonamides and trimethoprim) in animal manure by liquid chromatography–mass spectrometry. Journal of Chromatography A, 952, 111-120.

Hamscher, G., Sczesny, S., Höper, H., Nau, H. (2002): Determination of persistent tetracycline residues in soil fertilized with animal slurry by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. Analytical Chemistry, 74, 1509–1518.

Hao, C., Lissemore, L., Nguyen, B., Kleywegt, S., Yang, P., Solomon, K. (2006): Determination of pharmaceuticals in environmental waters by liquid chromatography/electrospray ionization/tandem mass spectrometry. Analytical and Bioanalytical Chemistry, 384, 505-513.

Halling-Sørensen, B., Lützhøft, H.C., Andersen, H.R., Ingerslev, F. (2000): Environmental risk assessment of antibiotics: Comparison of mecillinam, trimethoprim and ciprofloxacin. Journal of Antimicrobial Chemotherapy, 46, 53-58.

Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P.F., Ingerslev, F., Holten Liitzhofl, H.C., Jorgensen, S.E. (1998): Occurrence, fate and effects of pharmaceutical substances in the environment -- a review. Chemosphere, 36, 357-393.

Hamscher, G., Pawelzick, H.T., Sczesny, S., Nau, H., Hartung, J. (2003): Antibiotics in dust originating from a pig-fattening farm: A new source of health hazard for farmers? Environmental Health Perspectives, 111, 1590-1594.

Han, G.H., Hur, H.G., Kim, S.D. (2006): Ecotoxicological risk of pharmaceuticals from wastewater treatment plants in Korea: Occurrence and toxicity to *Daphnia magna.* Environmental Toxicology and Chemistry 25, 265-271.

Heberer, T. (2002a): Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: A review of recent research data. Toxicology Letters, 131, 5-17.

Heberer, T. (2002b): Tracking persistent pharmaceutical residues from municipal sewage to drinking water. Journal of Hydrology, 266, 175-189.

Hennessy, D.R. (1994): The disposition of antiparasitic drugs in relation to the development of resistance by parasites of livestock. Acta Tropica, 56, 125-141.

Hernando, M.D., Mezcua, M., Fernández-Alba, A.R., Barceló, D. (2006): Environmental risk assessment of pharmaceutical residues in wastewater effluents, surface waters and sediments. Talanta, 69, 334-342.

Hilton, M., Thomas, K. (2003): Determination of selected human pharmaceutical compounds in effluent and surface water samples by high-performance liquid chromatography–electrospray tandem mass spectrometry. Journal of Chromatography A, 1015, 129-141.

Horton, J. (2000): Albendazole: A review of anthelmintic efficacy and safety in humans. Parasitology, 121 113-132.

Hu, X.-Z., Wang, J.-X., Feng, Y.-Q. (2010): Determination of benzimidazole residues in edible animal food by polymer monolith microextraction combined with liquid chromatography-mass spectrometry. Journal of Agricultural and Food Chemistry, 58, 112-119.

Huttner, B., Harbarth, S. (2010): Variations in outpatient antimicrobial use between and within countries: An ongoing mystery. Infection, 38, 1-2.

Ikehata, K., Jodeiri Naghashkar, N., Gamal El-Din, M. (2006): Degradation of aqueous pharmaceuticals by ozonation and advanced oxidation processes. A Review, Ozone: Science and Engineering: The Journal of the International Ozone Association, 28, 353-414.

ICH Q2B (1996): Validation of analytical procedures: Methodology. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, Switzerland.

Isidori, M., Lavorgna, M., Nardelli, A., Parrella, A., Previtera, L., Rubino, M. (2005): Ecotoxicity of naproxen and its phototransformation products. Science of the Total Environment, 348, 93-101.

Ito, S., Tsukada, K. (2002): Matrix effect and correction by standard addition in quantitative liquid chromatographic-mass spectrometric analysis of diarrhetic shellfish poisoning toxins. Journal of Chromatography. A, 943, 39-46.

ISO11465 (1993): Soil quality – determination of dry matter and water content on a mass basis – gravimetric method.

ISO 10694 (1995): Soil quality – Determination of organic and total carbon after dry combustion (elementary analysis).

Issar, M., Nagaraja, N.V., Lal, J., Paliwal, J.K., Gupta, R.C. (1999): Determination of antifilarial compound UMF-078 and its metabolites in plasma by high-performance liquid chromatography. Journal of Chromatography B, Biomedical Sciences and Applications, 724, 147-155.

IUPAC. (2002): Quality assurance schemes for analytical laboratories * Harmonaized guidlines for -single laboratory validation of methods of analysis (IUPAC Technical Report) Pure and Applied Chemistry, 74, 835-855.

Jacobsen, a., Hallingsorensen, B., Ingerslev, F., Honorehansen, S. (2004): Simultaneous extraction of tetracycline, macrolide and sulfonamide antibiotics from agricultural soils using pressurised liquid extraction, followed by solid-phase extraction and liquid chromatography–tandem mass spectrometry. Journal of Chromatography A, 1038, 157-170.

Jones, O.A.H., Voulvoulis, N., Lester, J.N. (2002): Aquatic environmental assessment of the top 25 English prescription pharmaceuticals. Water Research, 36, 5013-5022.

Jørgensen, S. (2000): Drugs in the environment. Chemosphere, 40, 691-699.

Kan, C.A., Keukens, H.J., Tomassen, M.J. (1998): Flubendazole residues in eggs after oral administration to laying hens: Determination with reversed phase liquid chromatography. Analyst, 123, 2525-2527.

Karthikeyan, K.G., Meyer, M.T. (2006): Occurrence of antibiotics in wastewater treatment facilities in Wisconsin, USA. Science of the Total Environment, 361, 196-207.

Kasprzyk-Hordern, B., Dinsdale, R.M., Guwy, A.J. (2008): The occurrence of pharmaceuticals, personal care products, endocrine disruptors, and illicit drugs in surface water in South Wales, Water Research, 42, 3498-3518.

Keegan, J., O'Kennedy, R., Crooks, S., Elliott, C., Brandon, D., Danaher, M. (2011): Detection of benzimidazole carbamates and amino metabolites in liver by surface plasmon resonance-biosensor. Analytica Chimica Acta, 700, 41-48.

Kellar, Q.A.M.C., Gokbulut, C., Muzandu, K., Benchaoui, H. (2002): Fenbendazole pharmacokinitics, metabolism, and potentiation in horses. Pharmacology, 30, 1230-1239.

Khan, S.J., Ongerth, J.E. (2004): Modelling of pharmaceutical residues in Australian sewage by quantities of use and fugacity calculations. Chemosphere, 54, 355-367.

Kim, H.-J., Lee, D.S., Kwon, J.-H. (2010a): Sorption of benzimidazole anthelmintics to dissolved organic matter surrogates and sewage sludge. Chemosphere, 80, 256-262.

Kim, K.-R., Owens, G., Kwon, S.-I., So, K.-H., Lee, D.-B., Ok, Y.S. (2010b): Occurrence and environmental fate of veterinary antibiotics in the terrestrial environment. Water, Air, and Soil Pollution, 214, 163-174.

Kim, S.-C., Carlson, K. (2007): Quantification of human and veterinary antibiotics in water and sediment using SPE/LC/MS/MS. Analytical and Bioanalytical Chemistry, 387, 1301-1315.

Kim, S.-C., Carlson, K. (2005): LC–MS2 for quantifying trace amounts of pharmaceutical compounds in soil and sediment matrices. Trends in Analytical Chemistry, 24, 635-644.

Kim, S., Eichhorn, P., Jensen, J.N., Weber, A.S., Aga, D.S. (2005): Removal of antibiotics in wastewater: effect of hydraulic and solid retention times on the fate of tetracycline in the activated sludge process. Environmental Science and Technology, 39, 5816-5823.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T. (2002): Pharmaceuticals, hormones, and other organic wastewater contaminants in United State streams, 1999-2000: a national reconnaissance. Environmental Science and Technology, 36, 1202-1211.

Kreuzig, R. (2010): The reference manure concept for transformation tests of veterinary medicines and biocides in liquid manure. CLEAN - Soil, Air, Water, 38, 697-705.

Kreuzig, R., Schlag, P., Teigeler, J., Hartmann, C., Cvetkovic´, B. (2010a): Technical protocol for laboratory tests of transformation of veterinary medicinal products and biocides in liquid manures. UBA-Texte 45/07, ISSN 1862-4804. Umwelbundsamt, Dessau-Roßlau, 1-116, http://www.uba.de/uba-info-medien-e/3993.html, retrieved July 10, 2011.

Kreuzig, R., Blümlein, K., Höltge, S. (2007): Fate of the benzimidazole antiparasitics flubendazole and fenbendazole in manure and manured soils. CLEAN – Soil, Air, Water, 35, 488-494.

Kreuzig, R., Höltge, S., Heise, J., Schmanteck, I., Stein, F., Batarseh, M. (2007a): Veterinary medicinal products in manures and manured soils: Development of a technical protocol for laboratory tests. TUBA-Texte 45/07, ISSN 1862-4804. Umwelbundsamt, Dessau-Roßlau,1-142, http://www.umweltdaten.de/publikationen/fpdf-l/3343.pdf, retrieved July 10, 2011.

Kreuzig, R., Höltge, S. (2005): Investigation on fate of sulfadiazine in manured soil: Laboratory experiments and test pilot studies. Environmental Toxicology and Chemistry, 24, 777-781.

Kreuzig, R., Höltige, S., Brunotte, J., Berenzen, N., Wogram, J., Schulz, R. (2005): Test-plot studies on runoff of sulfonamides from manured soils after sprikler irrigation. Environmental Toxicology and Chemistry, 24, 771-776.

Kümmerer, K. (2009): The presence of pharmaceuticals in the environment due to human use - present knowledge and future challenges. Journal of Environmental Management, 90, 2354-2366.

Kümmerer, K. (2008): Pharmaceuticals in the environment: sources, fate, effects and risks. Springer-Verlag, Berlin Heidelberg, Germany.

Kümmerer, K. (2004): Resistance in the environment. The Journal of Antimicrobial Chemotherapy, 54, 311-320.

Lagerwerf, F.M., Dongen, W.D.V., Steenvoorden, R.J.J.M., Honing, M., Jonkman, J.H.G. (2000): Exploring the boundaries of bioanalytical quantitative LC-MS-MS. Trends in Analytical Chemistry, 19, 418-427.

Lanusse, C.E., Prichard, R.K. (1993): Relationship between pharmacological properties and clinical efficacy of ruminant anthelmintics. Veterinary Parasitology, 49, 123-158.

Larsson, D.G.J., de Pedro, C., Paxeus, N. (2007): Effluent from drug manufactures contains extremely high levels of pharmaceuticals. Journal of Hazardous Materials, 148, 751-755.

Li, C., Wu, Y.-L., Yang, T., Zhang, Y. (2011): Rapid simultaneous determination of eight benzimidazoles in animal feed by LC–MS–MS. Chromatographia, 73, 59-65.

Li, D., Yang, M., Hu, J., Ren, L., Zhang, Y., Li, K. (2008): Determination and fate of oxytetracycline and related compounds in oxytetracycline production wastewater and the receiving river. Environmental Toxicology and Chemistry, 27, 80-86.

Liang, H.R., Foltz, R.L., Meng, M., Bennett, P. (2003): Ionization enhancement in atmospheric pressure chemical ionization and suppression in electrospray ionization between target drugs and stable-isotope-labeled internal standards in quantitative liquid chromatography/tandem mass spectrometry. Rapid Communications in Mass spectrometry, 17, 2815-2821.

Lienert, J., Güdel, K., Escher, B.I. (2007): Screening method for ecotoxicological hazard assessment of 42 pharmaceuticals considering human metabolism and excretory routes. Environmental Science and Technology, 41, 4471-4478.

Lishman, L., Smyth, S.A., Sarafin, K., Kleywegt, S., Toito, J., Peart, T., Lee, B., Servos, M., Beland , M., Seto, P. (2006): Occurrence and reductions of pharmaceuticals and personal care products and estrogens by municipal wastewater treatment plants in Ontario, Canada. Science of the Total Environment, 367, 544-558.

Löffler, D., Ternes, T. (2003): Determination of acidic pharmaceuticals, antibiotics, and ivermectin in river sediment using liquid chromatography–tandem mass spectrometry. Journal of Chromatography A, 1021, 133-144.

Long, A.R., Hsieh, L.C., Malbrough, M.S., Short, C.R., Barker, S.A. (1989): Multiresidue method for isolation and liquid chromatographic determination of seven benzimidazole anthelmintics in milk. Journal of the Association of Official Analytical Chemists, 5.

Long, A.R., Hsieh, L.C., Malbrough, M.S., Short, C.R., Barker, S.A. (1990): Matrix solid phase dispersion isolation and liquid chromatographic determination of five benzimidazole anthelmintics inpork muscle tissue. Journal of Food Composition and Analysis, 3, 20-26.

Loraine, G.A., Pettigrove, M.E. (2006): Seasonal variations in concentrations of pharmaceuticals and personal care products in drinking water and reclaimed wastewater in southern California. Environmental Science and Technology, 40, 687-695.

Lunestad, B.T., Goksayr, J. (1990): Reduction in the antibacterial effect of oxytetracycline in sea water by complex formation with magnesium and calcium. Diseases of Aquatic Organisms, 9, 67-72.

Mallet, C.R., Lu, Z., Mazzeo, J.R. (2004): A study of ion suppression effects in electrospray ionization from mobile phase additives and solid-phase extracts. Rapid Communications in Mass Spectrometry, 18, 49-58.

Maraschiello, C., Cusidó, E., Abellán, M., Vilageliu, J. (2001): Validation of an analytical procedure for the determination of the fluoroquinolone ofloxacin in chicken tissues. Journal of Chromatography. B, Biomedical Sciences and Applications, 754, 311-318.

March, R.E. (1997): Special feature: An Introduction to Quadrupole Ion Trap Mass Spectrometry. Journal of Mass Spectrometry, 32, 351-369.

March, R.E., TODD, J.F.J.(2005):Quadrupole ion trap mass spectrometry. John Wiley and Sons, Inc., Hoboken, New Jersey (Chapter 2).

Merkel, D. (2005): Personal communication. 301-305.

Marti, A. M., Moose, A. E., KOCH, H. (1990): Determination of benzimidazole anthelmintics in meat samples. Journal of Chromatography, 498, 145-157.

Miao, X.-S., Bishay, F., Chen, M., Metcalfe, C.D. (2004): Occurrence of antimicrobials in the final effluents of wastewater treatment plants in Canada. Environmental Science and Technology, 38, 3533-3541.

Miège, C., Favier, M., Brosse, C., Canler, J.-P., Coquery, M. (2006): Occurrence of betablockers in effluents of wastewater treatment plants from the Lyon area (France) and risk assessment for the downstream rivers. Talanta, 70, 739-744.

Moreno, L., Imperiale, F., Mottier, L., Alvarez, L., Lanusse, C. (2005): Comparison of milk residue profiles after oral and subcutaneous administration of benzimidazole anthelmintics to dairy cows. Analytica Chimica Acta, 536, 91-99.

Moreno, L., Alvarez, L., Mottier, L., Virkel, G., Bruni, S.S., Lanusse, C. (2004): Integrated pharmacological assessment of flubendazole potential for use in sheep: Disposition kinetics, liver metabolism, and parasite diffusion ability. Journal of Veterinary Pharmacology and Therapeutics, 27, 299-308.

Morovján, G., Csokán, P., Makranszki, L., Abdellah-Nagy, E.A., Tóth, K. (1998): Determination of fenbendazole, praziquantel, and pyrantel pamoate in dog plasma by high-performance liquid chromatography. Journal of Chromatography A, 797, 237-244.

Mottier, L., Alvarez, L., Lanusse, C. (2003): Quantitative chromatographic determination of several benzimidazole anthelmintic molecules in parasite material. Journal of Chromatography B, 798, 117-125.

Msagati, T.A.M., Nindi, M.M. (2006): Comparative study of sample preparation methods; supported liquid membrane and solid phase extraction in the determination of benzimidazole anthelmintics in biological matrices by liquid chromatography-electrospray-mass spectrometry. Talanta, 69, 243-250.

Msagati, T.A.M., Nindi, M.M. (2001): Determination of benzimidazole anthelmintic compounds by supported liquid membrane extraction and liquid chromatography. Journal of Separation Science, 24, 606-614.

Müller, C. (2004): Entwicklung von Screeningverfahren für Arzneistoffe und Metaboliten mittels LC-MS und LC-MS/MS. PhD Thesis, Albert-Ludwigs-Universität Freiburg

Murray, M., Hudson, A.M., Yassa, V. (1992): Hepatic microsomal metabolism of the anthelmintic benzimidazole fenbendazole: Enhanced inhibition of cytochrome P450 reactions by oxidized metabolites of the drug. Chemical Research in Toxicology, 5, 60-66.

Nakada, N., Tanishima, T., Shinohara, H., Kiri, K., Takada, H. (2006): Pharmaceutical chemicals and endocrine disrupters in municipal wastewater in Tokyo and their removal during activated sludge treatment. Water Research, 40, 3297-3303.

Nash, J.P., Kime, D.E., Van der Ven, L.T.M., Wester, P.W., Brion, F., Maack, G., Stahlschmidt-Allner, P., Tyler, C.R. (2004): Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. Environmental Health Perspectives, 112, 1725-1733.

Nerín, C., Cacho, J., Tornés, a.R., Echarri, I. (1994): Some observations on the standard addition procedure in gas chromatographic analysis. Journal of Chromatography A, 672, 159-165.

Nerenberg, C., Runkel, R.A., Matin, S.B. (1978): Radioimmunoassay of oxfendazole in bovine, equine, or canine plasma or serum. Journal of Pharmaceutical Sciences, 67, 1553-1557.

Nikolaou, A., Meric, S., Fatta, D. (2007): Occurrence patterns of pharmaceuticals in water and wastewater environments. Analytical and Bioanalytical Chemistry, 387, 1225-1234.

Nobilis, M., Jira, T., Lísa, M., Holcapek, M., Szotáková, B., Lamka, J., Skálová, L. (2007): Achiral and chiral high-performance liquid chromatographic determination of flubendazole and its metabolites in biomatrices using UV photodiode-array and mass spectrometric detection. Journal of Chromatography A, 1149, 112-120.

Oaks, J.L., Gilbert, M., Virani, M.Z., Watson, R.T., Meteyer, C.U., Rideout, B.A., Shivaprasad, H.L., Ahmed, S., Chaudhry, M.J.I., Arshad, M., Mahmood, S., Ali, A., Khan, A.A. (2004): Diclofenac residues as the cause of vulture population decline in Pakistan. Nature, 427, 630-633.

OECD (2002): Guideline for the testing of chemicals. Aerobic and anaerobic degradation in soil. Guideline 307. Paris, France, 1–17.

OECD (2000): Guideline for testing of chemicals. Adsorption-desorption using a batch equilibrium method. 106, 1-45.

Ortelli, D., Cognard, E., Jan, P., Edder, P. (2009): Comprehensive fast multiresidue screening of 150 veterinary drugs in milk by ultra-performance liquid chromatography coupled to time of flight mass spectrometry. Journal of Chromatography B, Analytical Technologies in the Biomedical and Life Sciences, 877, 2363-2374.

Peters, F.T., Drummer, O.H., Musshoff, F. (2007): Validation of new methods. Forensic Science International, 165, 216-224.

Pfeifer, T., Tuerk, J., Bester, K., Spiteller, M. (2002): Determination of selected sulfonamide antibiotics and trimethoprim in manure by electrospray and atmospheric pressure chemical ionization tandem mass spectrometry. Rapid Communications in Mass Spectrometry, 16, 663-669.

Poiger, T., Buser, H.R., Müller, M.D. (2001): Photodegradation of the pharmaceutical drug diclofenac in a lake: Pathway, field measurements, and mathematical modeling. Environmental Toxicology and Chemistry, 20, 256-263.

Pomati, F., Castiglioni, S., Zuccato, E., Fanelli, R., Vigetti, D., Rossetti, C., Calamari, D. (2006): Effects of a complex mixture of therapeutic drugs at environmental levels on human embryonic cells. Environmental Science and Technology, 40, 2442-2447.

Ramanathan, S., Nair, N.K., Mansor, S.M., Navaratnam, V. (1994): Determination of the antifilarial drug UMF-078 and its metabolites UMF-060 and flubendazole in whole

blood using high-performance liquid chromatography. Journal of Chromatography B, Biomedical Applications, 655, 269-273.

Rose, M.D. (1999): A method for the separation of residues of nine compounds in cattle liver related to treatment with oxfendazole. Analyst, 124, 1023-1026.

Rousseau, A., Gillotin, F., Chiap, P., Crommen, J., Fillet, M., Servais, A.-C. (2010): Association of two single-isomer anionic CD in NACE for the chiral and achiral separation of fenbendazole, its sulphoxide and sulphone metabolites: Application to their determination after in vitro metabolism. Electrophoresis, 31, 1482-1487.

Samuelsen, O.B. (1989): Degradation of oxytetracycline in seawater at two different temperatures and light Intensities, and the persistence of oxytetracycline in the sediment from a fish farm. Aquaculture, 83, 7-16.

Sarmah, A.K., Meyer, M.T., Boxall, A.B.A. (2006): A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere, 65, 725-759.

Schulze, T., Weiss, S., Schymanski, E., von der Ohe, P.C., Schmitt-Jansen, M., Altenburger, R., Streck, G., Brack, W. (2010): Identification of a phytotoxic photo-transformation product of diclofenac using effect-directed analysis. Environmental Pollution, 158, 1461-1466.

Schwab, B.W., Hayes, E.P., Fiori, J.M., Mastrocco, F.J., Roden, N.M., Cragin, D., Meyerhoff, R.D., D'Aco, V.J., Anderson, P.D. (2005a): Human pharmaceuticals in US surface waters: A human health risk assessment. Regulatory Toxicology and Pharmacology, 42, 296-312.

Schwab, A.E., Boakye, D.A., Kyelem, D., Prichard, R.K. (2005b): Detection of benzimidazole resistance-associated mutations in the filarial nematode *Wuchereria bancrofti* and evidence for selection by albendazole and ivermectin combination treatment. American Journal of Tropical Medicine and Hygene, 37, 234-238.

Schwarzenbach, R.P., Escher, B.I., Fenner, K., Hofstetter, T.B., Johnson, C.A., von Gunten, U., Wehrli, B. (2006): The challenge of micropollutants in aquatic systems. Science, 313, 1072-1077.

Schlüsener, M.P., von Arb, M.a., Bester, K. (2006): Elimination of macrolides, tiamulin, and salinomycin during manure storage. Archives of Environmental Contamination and Toxicology, 51, 21-28.

Shah, V.P., Midha, K.K., Findlay, J.W., Hill, H.M., Hulse, J.D., McGilveray, I.J., McKay, G., Miller, K.J., Patnaik, R.N., Powell, M.L., Tonelli, a., Viswanathan, C.T., Yacobi, a. (2000): Bioanalytical method validation--a revisit with a decade of progress. Pharmaceutical Research, 17, 1551-1557.

Shah VP, M.K., Dighe S, McGilveray IJ, Skelly JP, Yacobi A, Layloff T, Viswanathan CT, Cook CE, McDowall RD, pittman K A, Spector S. (1992): Analytical methods validation: Bioavialability, bioequivqlence and phramacokinitic studies. Pharmaceutical Research, 9, 588-592.

Souverain, S., Rudaz, S., Veuthey, J.-L. (2004): Matrix effect in LC-ESI-MS and LC-APCI-MS with off-line and on-line extraction procedures. Journal of Chromatography A, 1058, 61-66.

Stroobant, V., Hoffmann, E.(2007): Mass spectrometry principles and applications, John Wiley & Sons Ltd, West Sussex PO19 8SQ, England.

Su, S.-C., Chou, H.-H., Chang, P.-C., Liu, C.-H., Chou, S.-S. (2004): Simultaneous determination of febantel , fenbendazole , oxfendazole and oxfendazole sulfone in livestock by matrix solid phase dispersion extraction technique and HPLC. Journal of Food and Drug Analysis, 12, 244-253.

Sørensen, L.K., Hansen, H. (1998): Determination of fenbendazole and its metabolites in trout by a high-performance liquid chromatographic method. Analyst, 123, 2559-2562.

Spasov, A.A., Yozhitsa, I.N., Bugaeva, L.I., Anisimova, V.A. (1999): Benzimidazole derivatives: Spectrum of pharmacological activity and toxicological properties. Journal of Pharmaceutical Chemistry, 33, 232-243.

Stoob, K., Singer, H.P., Mueller, S.R., Schwarzenbach, R.P., Stamm, C.H. (2007): Dissipation and transport of veterinary sulfonamide antibiotics after manure application to grassland in a small catchment. Environmental Science and Technology, 41, 7349-7355.

Straub, J.O., Stewat, K.M. (2007): Determination and probabilistic acute based environmental risk assessment for naproxen for western Europe. Environmental Toxicology and Chemistry, 26, 795-806.

Stumpf, M., Ternes, T.A., Wilken, R.D., Rodrigues, S.V., Baumann, W. (1999): Polar drug residues in sewage and natural waters in the state of Rio de Janeiro, Brazil. Science of the Total Environment, 225, 135-141.

Stürchler, D. (1987): Parasitic diseases of the small intestinal tract. Baillière's Clinical Gastroenterology, 1, 397-424.

Taylor, P.J. (2005): Matrix effects: The Achilles heel of quantitative high-performance liquid chromatography-electrospray-tandem mass spectrometry. Clinical Biochemistry, 38, 328-334.

Ternes, T.A. (1998): Occurrence of drugs in German sewage treatment plants and rivers. Water Research, 32, 3245-3260..

Ternes, T.A., Bonerz, M., Herrmann, N., Teiser, B., Andersen, H.R. (2007): Irrigation of treated wastewater in Braunschweig, Germany: An option to remove pharmaceuticals and musk fragrances. Chemosphere, 66, 894-904.

Ternes, T.A., Meisenheimer, M., McDowell, D., Sacher, F., Brauch, H.-J., Haist-Gulde, B., Preuss, G., Wilme, U., Zulei-Seibert, N. (2002): Removal of pharmaceuticals during drinking water treatment. Environmental Science and Technology, 36, 3855-3863.

Ternes, T.A., Stumpf, M., Mueller, J., Haberer, K., Wilken, R.D., Servos, M. (1999): Behavior and occurrence of estrogens in municipal sewage treatment plants -- I. Investigations in Germany, Canada, and Brazil. The Science of the Total Environment, 225, 81-90.

Thiele-Bruhn, S., Beck, I.-C. (2005): Effects of sulfonamide and tetracycline antibiotics on soil microbial activity and microbial biomass. Chemosphere, 59, 457-465.

Thurman, E.M., Ferrer, I., Barceló, D. (2001): Choosing between atmospheric pressure chemical ionization and electrospray ionization interfaces for the HPLC/MS analysis of pesticides. Analytical chemistry, 73, 5441-5449.

Tolls, J. (2001): Critical review sorption of veterinary pharmaceuticals in soils. Environmental Science and Technology, 35, 3397-3406.

Townsend, L.B., Wise, D.S. (1990): The synthesis and chemistry of certain anthelmintic benzimidazoles. Parasitology Today, 6, 107-112.

Trautwein, C., Kümmerer, K., Metzger, J.W. (2008): Aerobic biodegradability of the calcium channel antagonist verapamil and identification of a microbial dead-end transformation product studied by LC-MS/MS. Chemosphere, 72, 442-450.

Turiel, E., Bordin, G., Rodrıguez, A.R. (2003): Trace enrichment of (fluoro) quinolone antibiotics in surface waters by solid-phase extraction and their determination by liquid chromatography – ultraviolet detection. Water, 1008, 145-155.

United States Environmental Protection Agency (1984). Definition and procedure for the determination of the method detection limit. Appendix B to part 136.

Van De Steene, J.C., Lambert, W.E. (2008): Validation of a solid-phase extraction and liquid chromatography-electrospray tandem mass spectrometric method for the determination of nine basic pharmaceuticals in wastewater and surface water samples. Journal of Chromatography A, 1182, 153-160.

Van de Steene, J.C., Mortier, K.A., Lambert, W.E. (2006): Tackling matrix effects during development of a liquid chromatographic-electrospray ionisation tandem mass spectrometric analysis of nine basic pharmaceuticals in aqueous environmental samples. Journal of Chromatography A, 1123, 71-81.

Van Leemput, L., Heykants, J. (1991): Unpublished report. Submitted to FAO by Janssen Pharmaceutica.

Weigel, S., Kallenborn, R., Huhnerfuss, H. (2004): Simultaneous solid-phase extraction of acidic, neutral and basic pharmaceuticals from aqueous samples at ambient (neutral) pH and their determination by gas chromatography–mass spectrometry. Journal of Chromatography A, 1023, 183-195.

Weigel, S., Kuhlmann, J., Hühnerfuss, H. (2002): Drugs and personal care products as ubiquitous pollutants: Occurrence and distribution of clofibric acid, caffeine and DEET in the North Sea. Science of the Total Environment, 295, 131-141.

Weiss, K., Schüssler, W., Porzelt, M. (2008): Sulfamethazine and flubendazole in seepage water after the sprinkling of manured areas. Chemosphere, 72, 1292-1297.

Westerhoff, P., Yoon, Y., Snyder, S., Wert, E. (2005): Fate of endocrine-disruptor, pharmaceutical, and personal care product chemicals during simulated drinking water treatment processes. Environmental Science and Technology, 39, 6649-6663.

WHO. (1991): Evaluation of certain veterinary drug residues in food, Thirty-eighth Report of the Joint FAO/WHO Expert Committee on Food Additives, Technical Report Series 815, World Health Organization, Geneva.

WHO. (1993): Evaluation of certain veterinary drug residues in food, Fortieth Meeting of the Joint FAO/WHO Expert Committee on Food Additives,Food Technical Report Series 832, Agriculture Organization of the United Nations, Geneva.

Yang, S., Cha, J., Carlson, K. (2004): Quantitative determination of trace concentrations of tetracycline and sulfonamide antibiotics in surface water using solid-phase extraction and liquid chromatography/ion trap tandem. Rapid Communications in Mass Spectrometry, 18, 2131-2145.

Yokley, R.A., Mayer, L.C., Rezaaiyan, R., Manuli, M.E., Cheung, M.W. (2000): Analytical method for the determination of cyromazine and melamine residues in soil using LC-UV and GC-MSD. Journal of Agricultural and Food Chemistry, 48, 3352-3358.

Younes, M. (1999): Specific issues in health assessment international. Science, 39, 1253-1257.

Zhou, J., Xue, X., Li, Y., Zhang, J., Chen, F., Wu, L., Chen, L., Zhao, J. (2009): Multiresidue determination of tetracycline antibiotics in propolis by using HPLC-UV detection with ultrasonic-assisted extraction and two-step solid phase extraction. Food Chemistry, 115, 1074-1080.

Zhu, J., Snow, D.D., Cassada, D.A., Monson, S.J., Spalding, R.F. (2001): Analysis of oxytetracycline, tetracycline, and chlortetracycline in water using solid-phase extraction and liquid chromatography-tandem mass spectrometry. Journal of Chromatography A, 928, 177-186.

Wisconsin Department of Natural Resources, Laboratory Certification Program (1996): Analytical detection limit guidance and laboratory guide for determining method detection limits, PUBL-TS-056-96.

APPENDIX

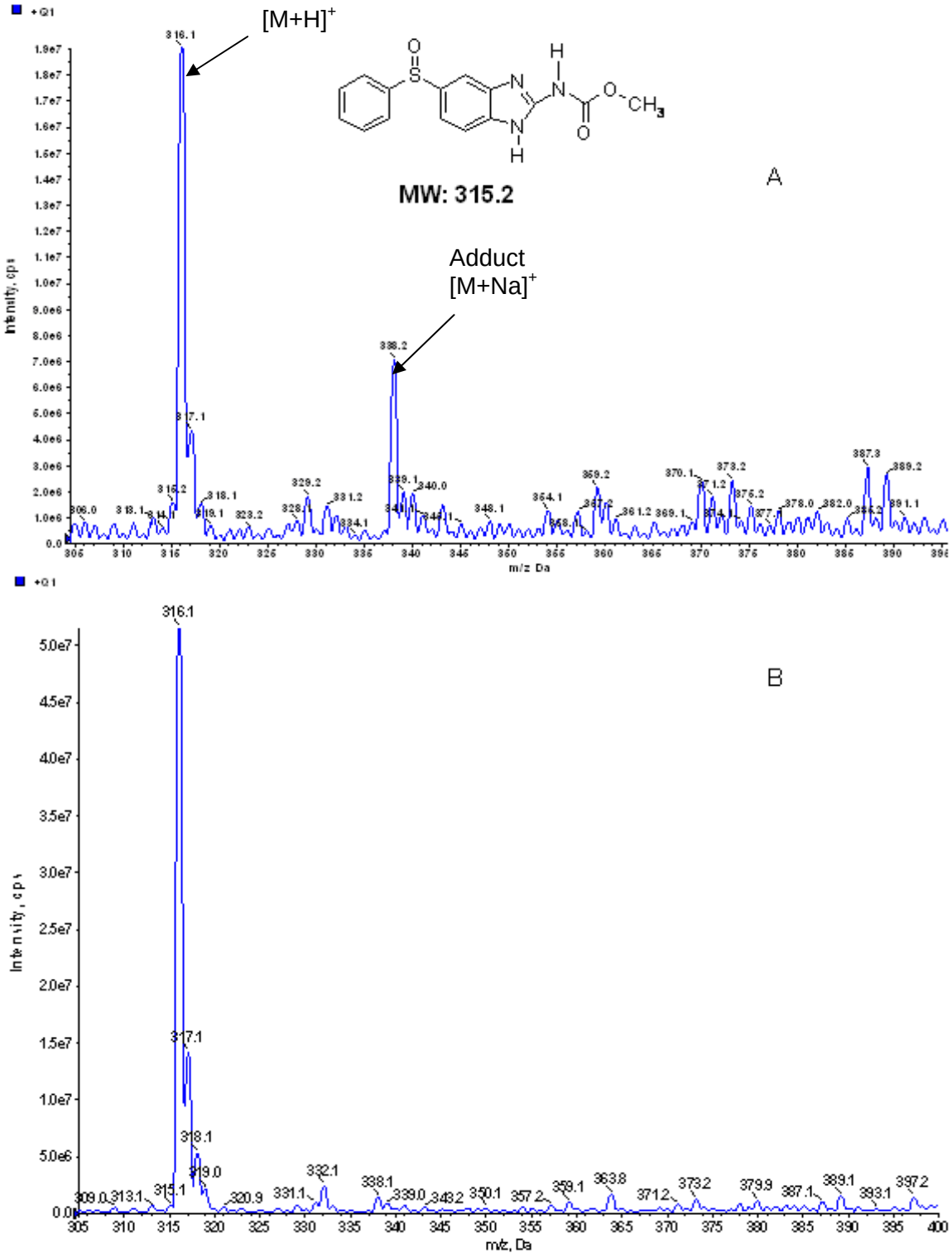


Figure A1: Mass spectra of fenbendazole sulfoxide and fenbendazole sulfone recorded in full scan mode showing A: the precursor ion with the corresponding sodium adduct in methanol, B: sodium adduct was avoided by adding formic acid in methanol using electrospray source in positive ionization mode.

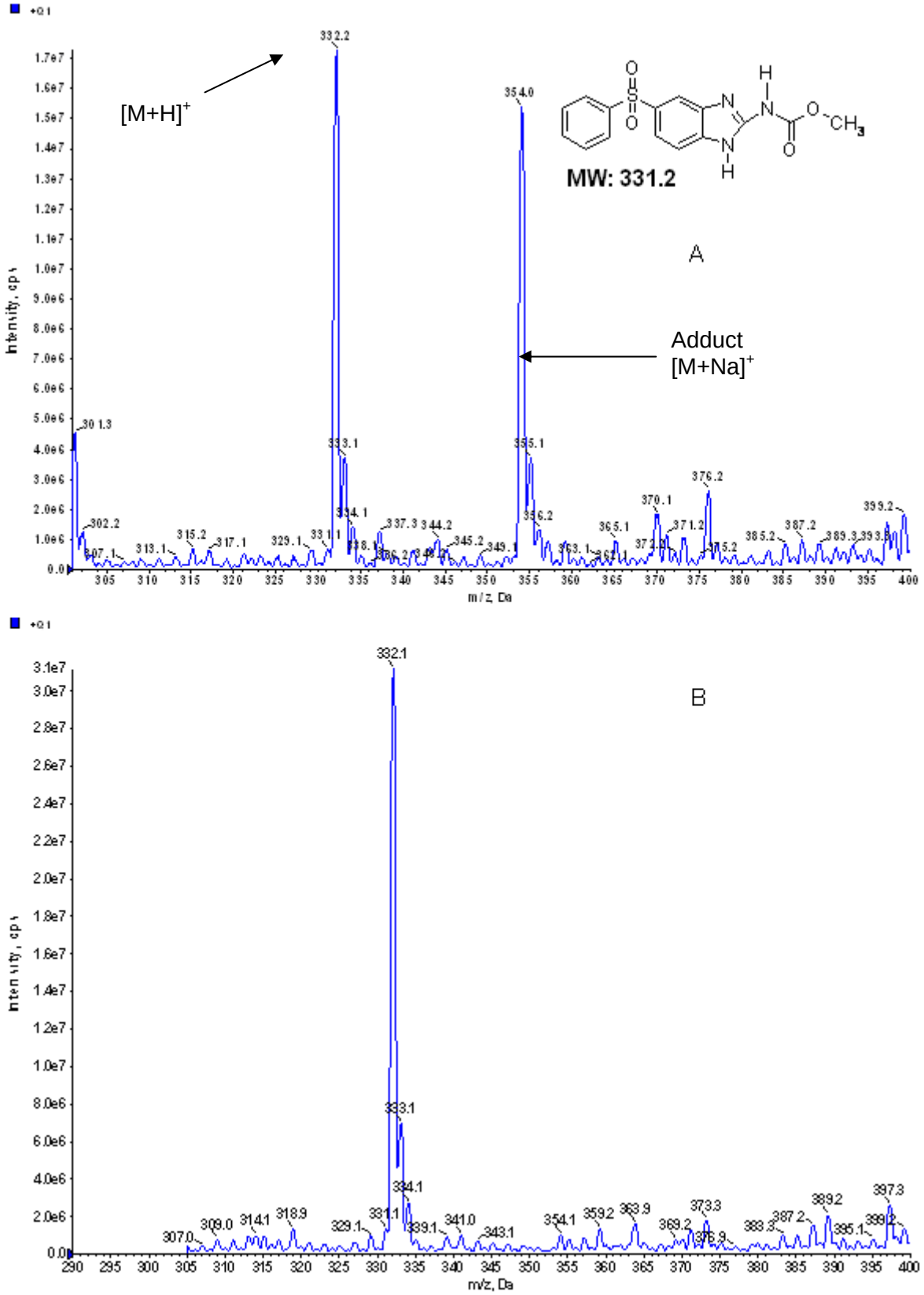


Figure A1: Continued.

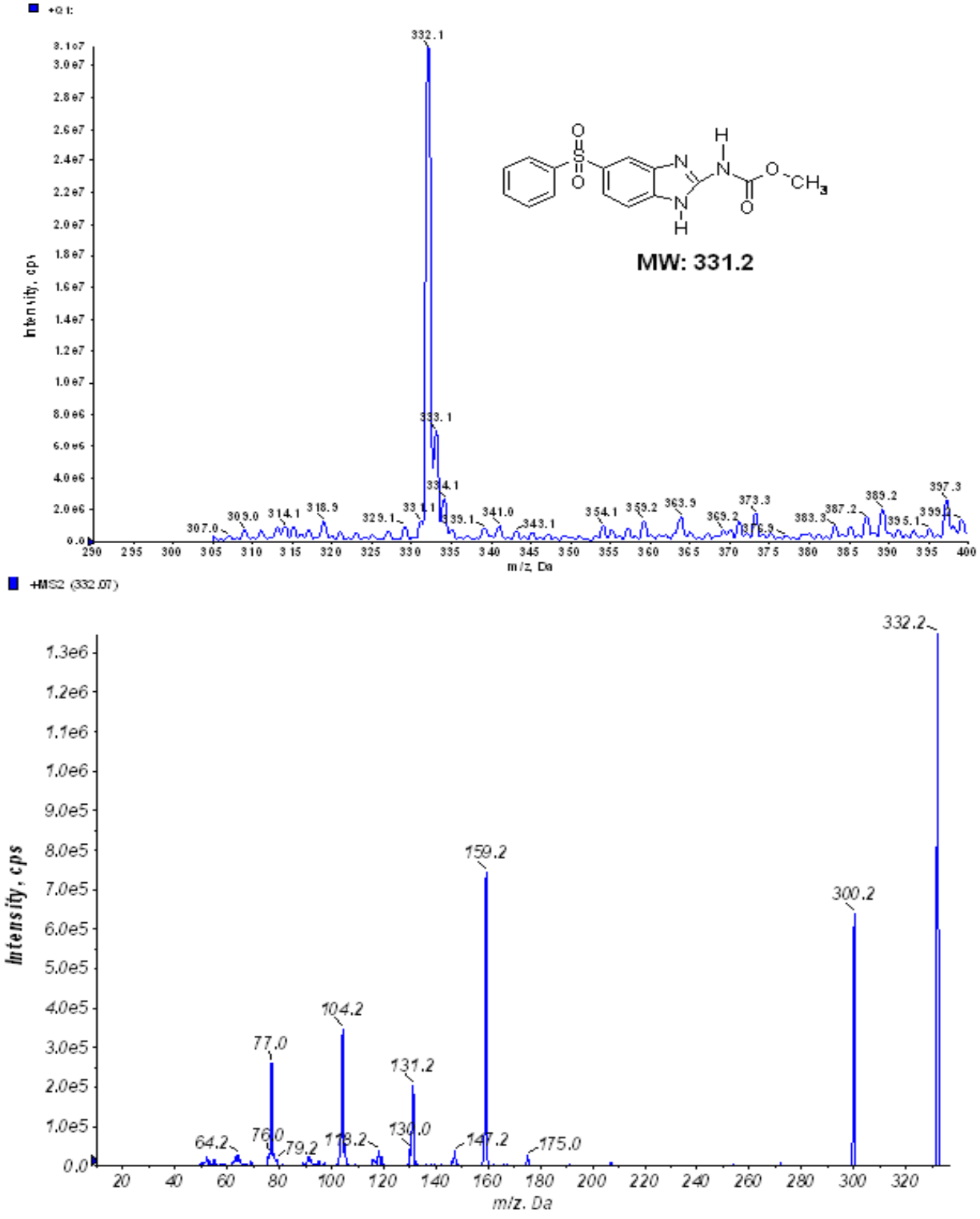


Figure A2: Mass spectra of fenbendazole sulfone and flubendazole metabolites (FLU-M2, FLU-M3, FLU-M4, FLU-M5 and FLU-M6) A: Q1 (MS) showing the precursor ions, B: product ion (MS/MS) showing the different fragments of the target compounds in positive electrospray ionization mode (ESI+).

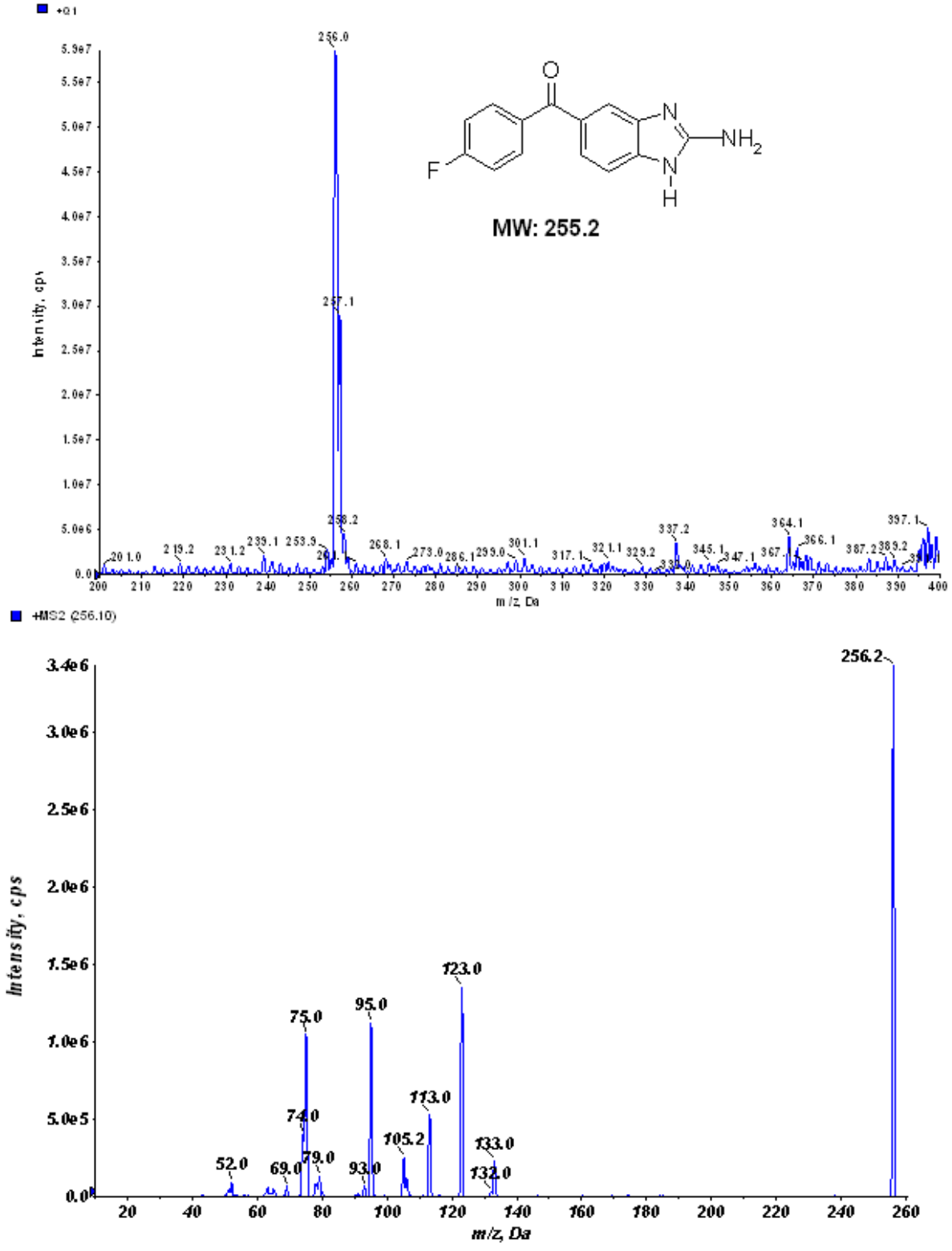


Figure A2: Continued.

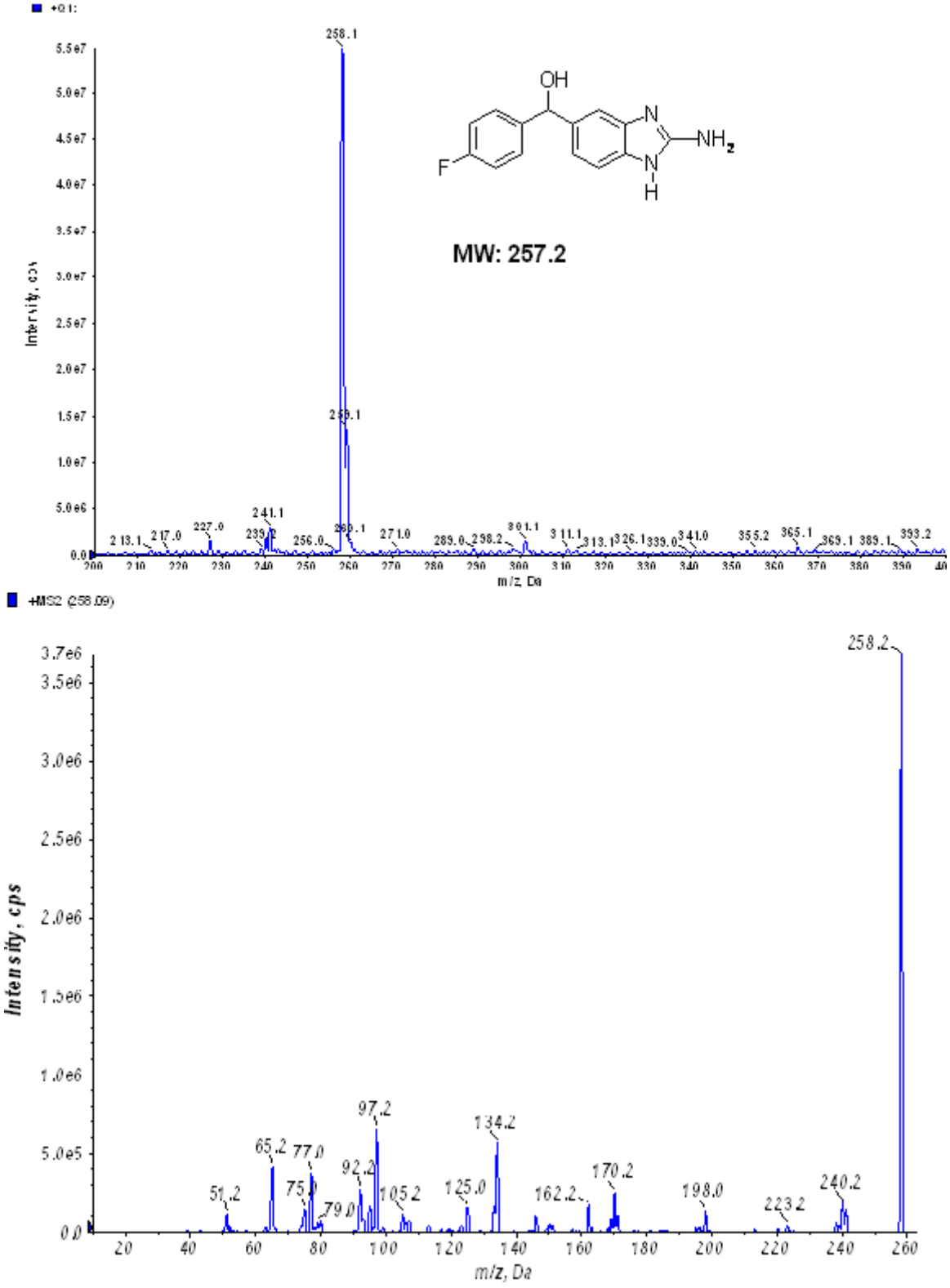


Figure A2: Continued.

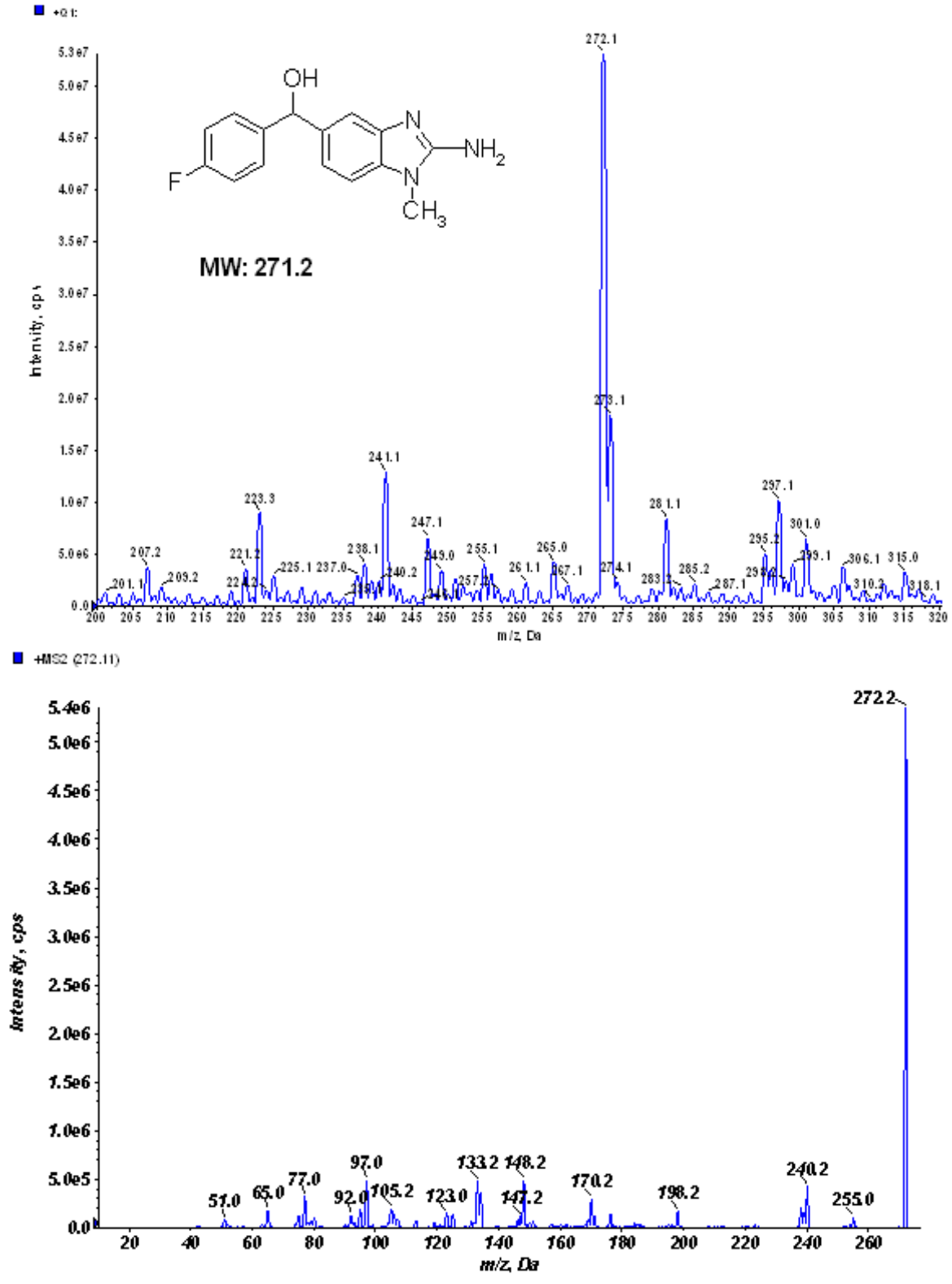


Figure A2: Continued.

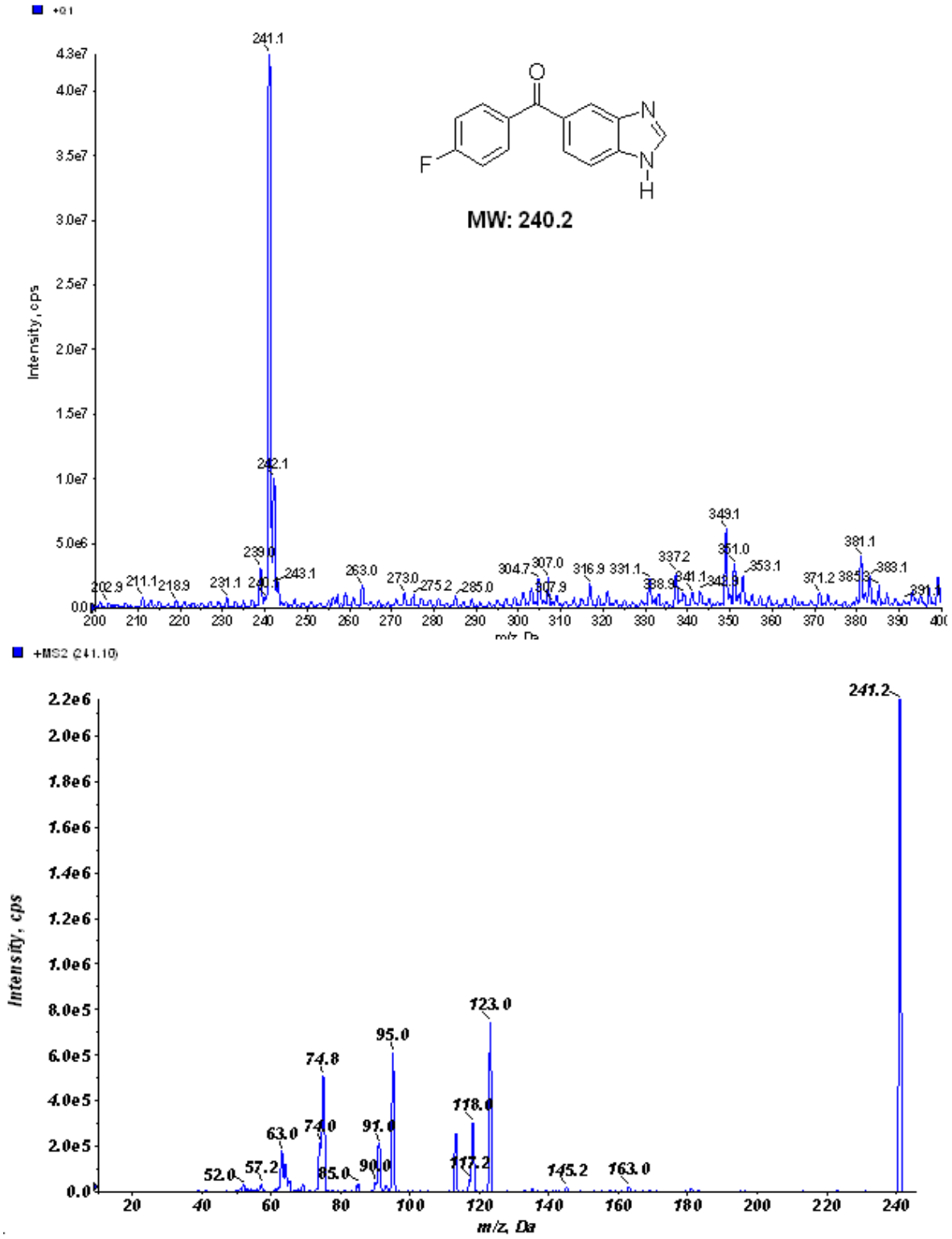


Figure A2: Continued.

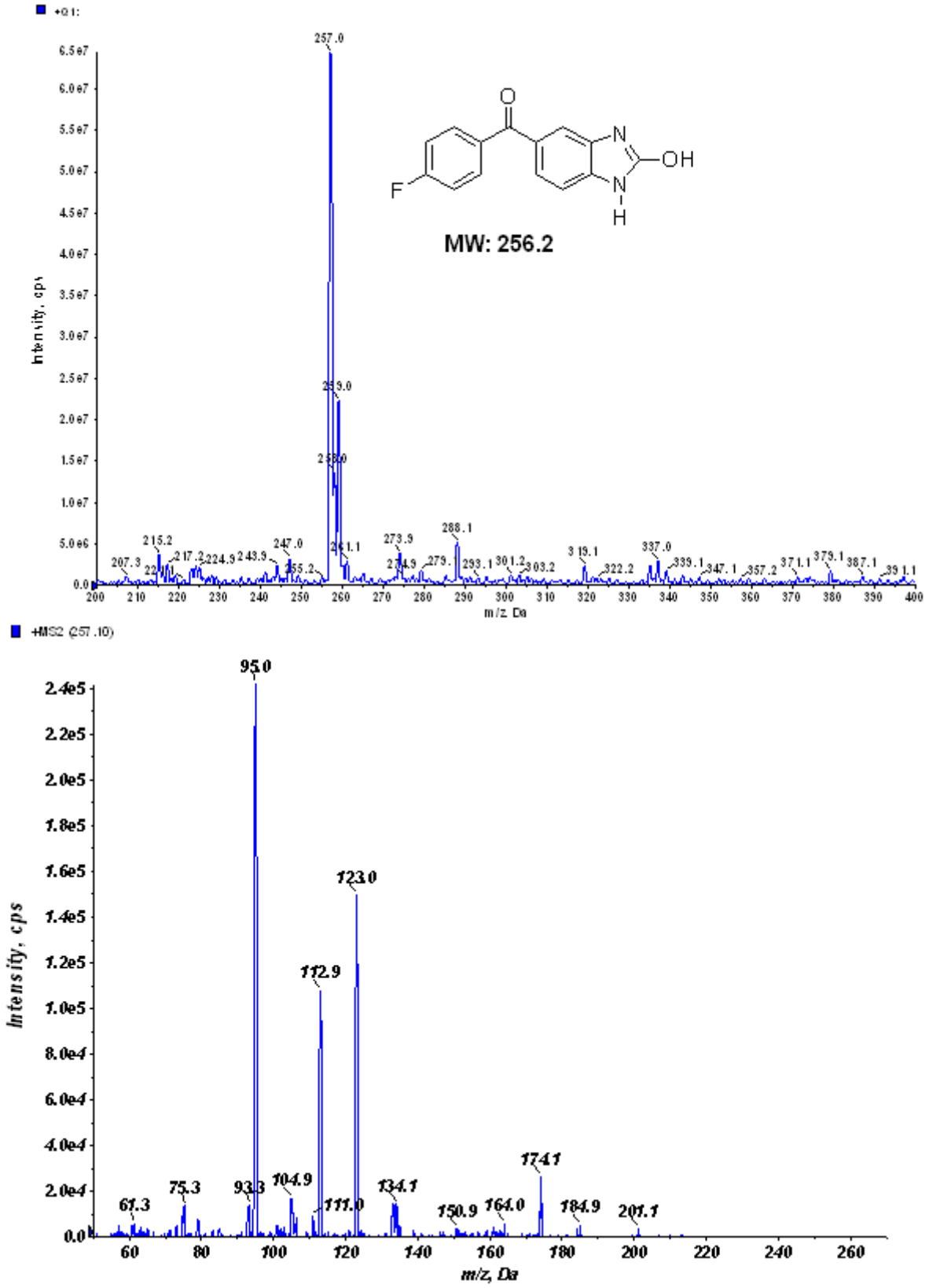


Figure A2: Continued.

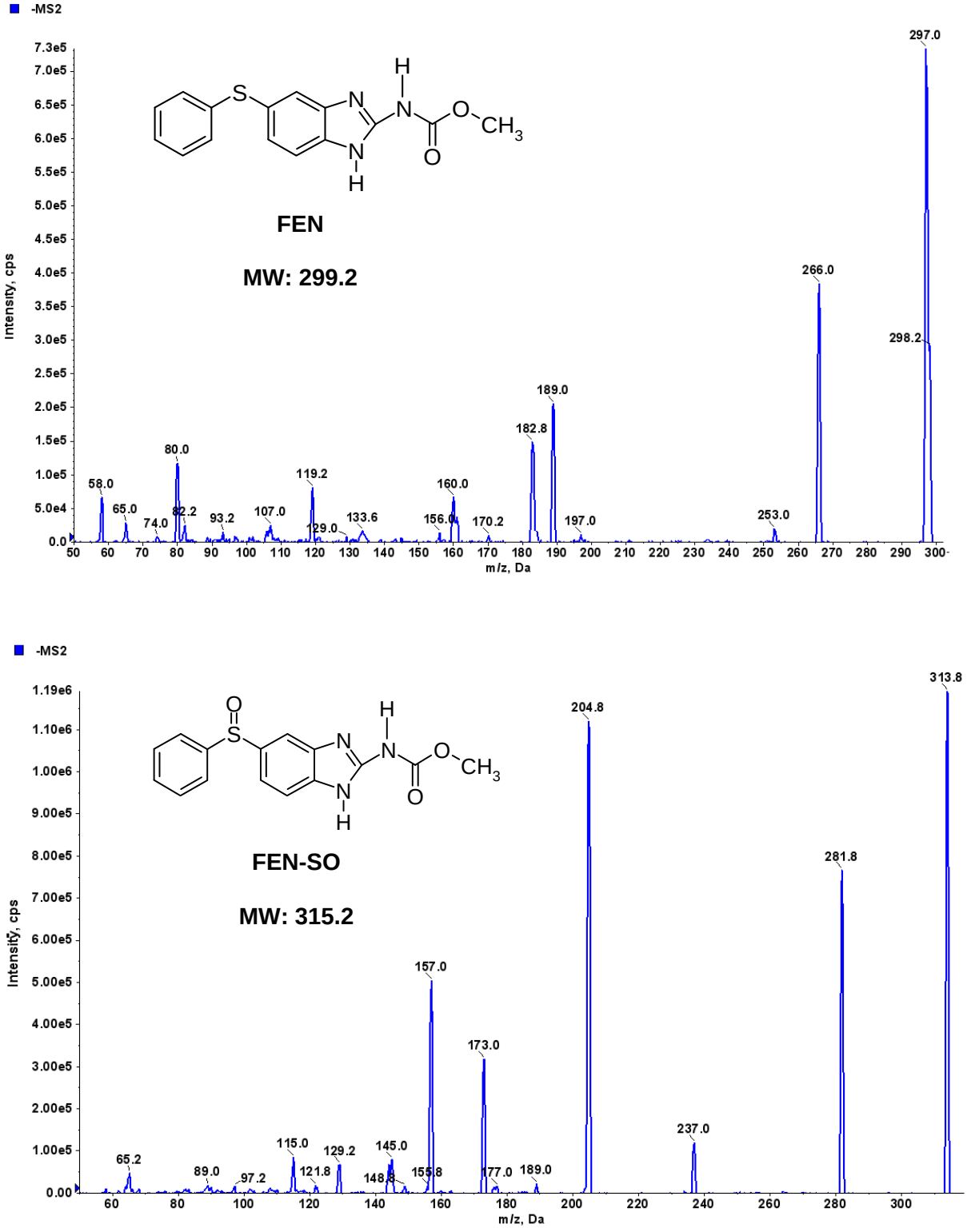
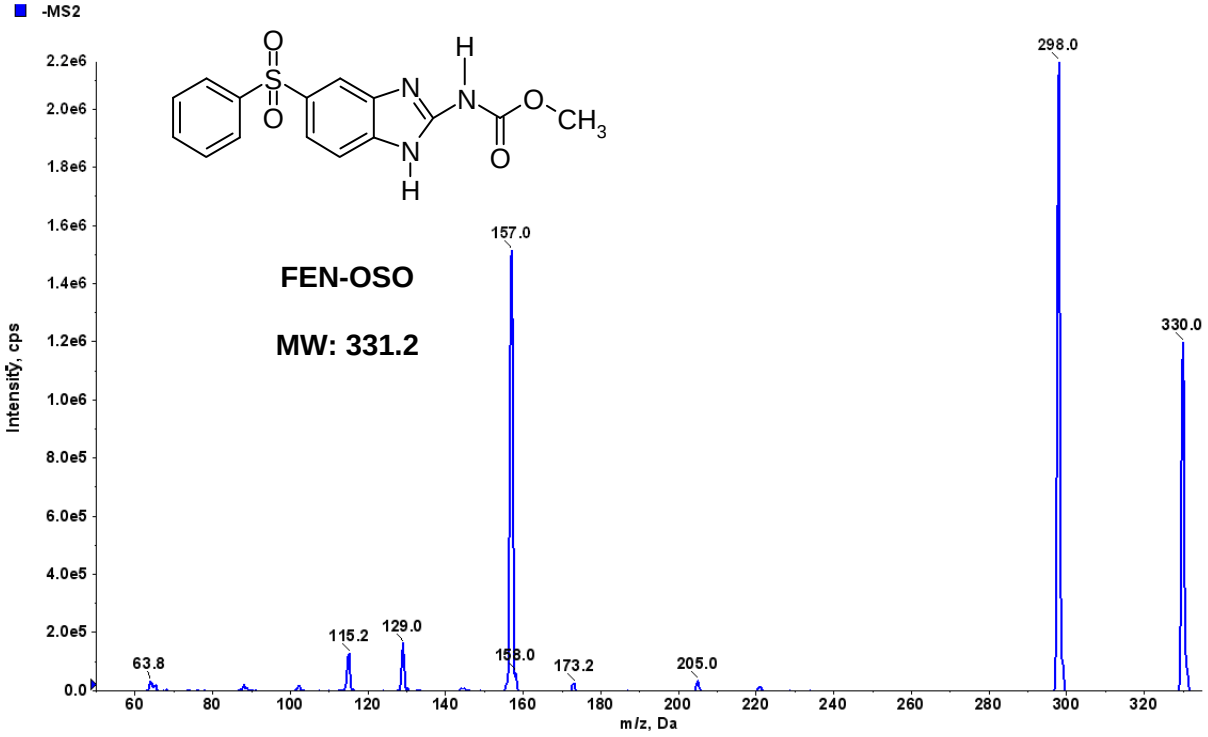


Figure A3: Product ion spectra of target compounds in negative ionization mode using electrospray ionisation source (ESI-).



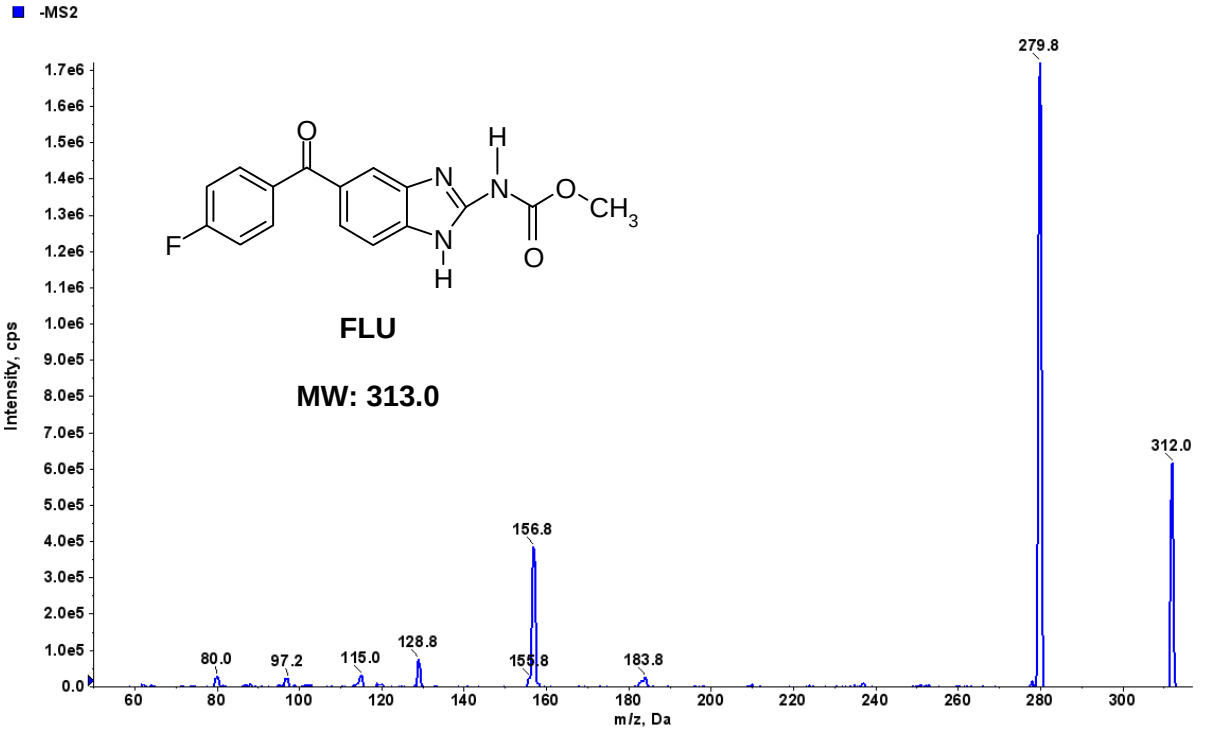
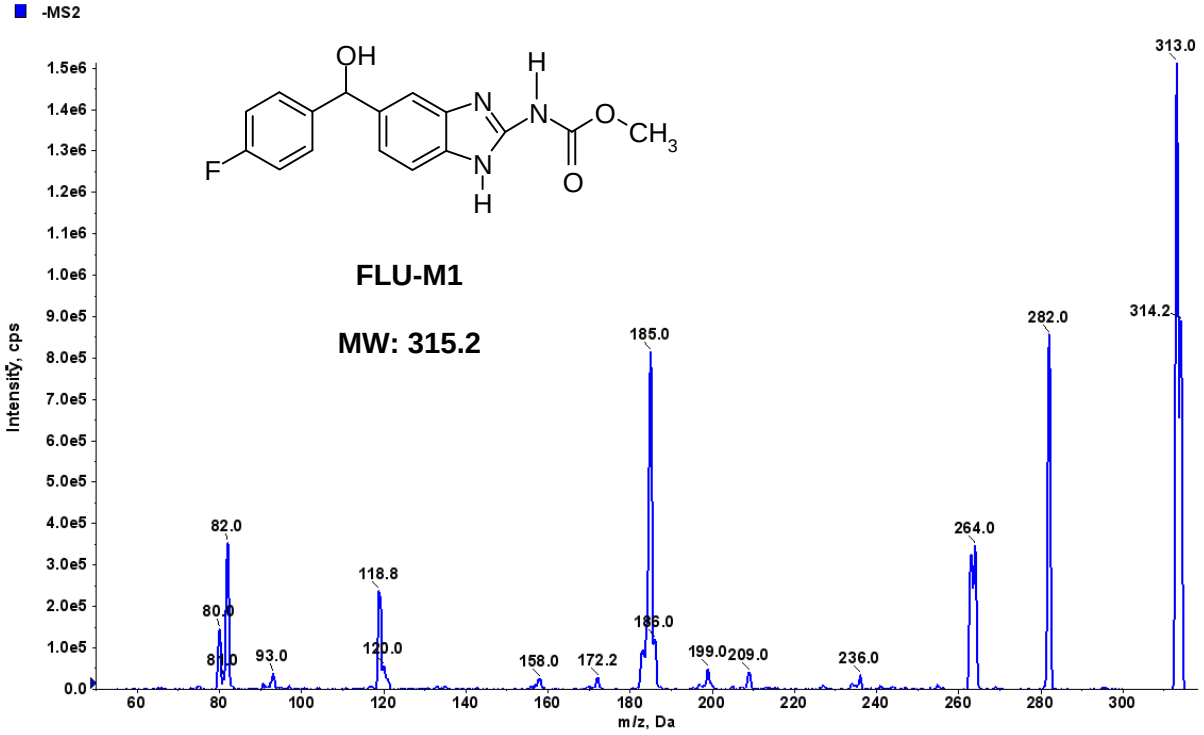


Figure A3: Continued.



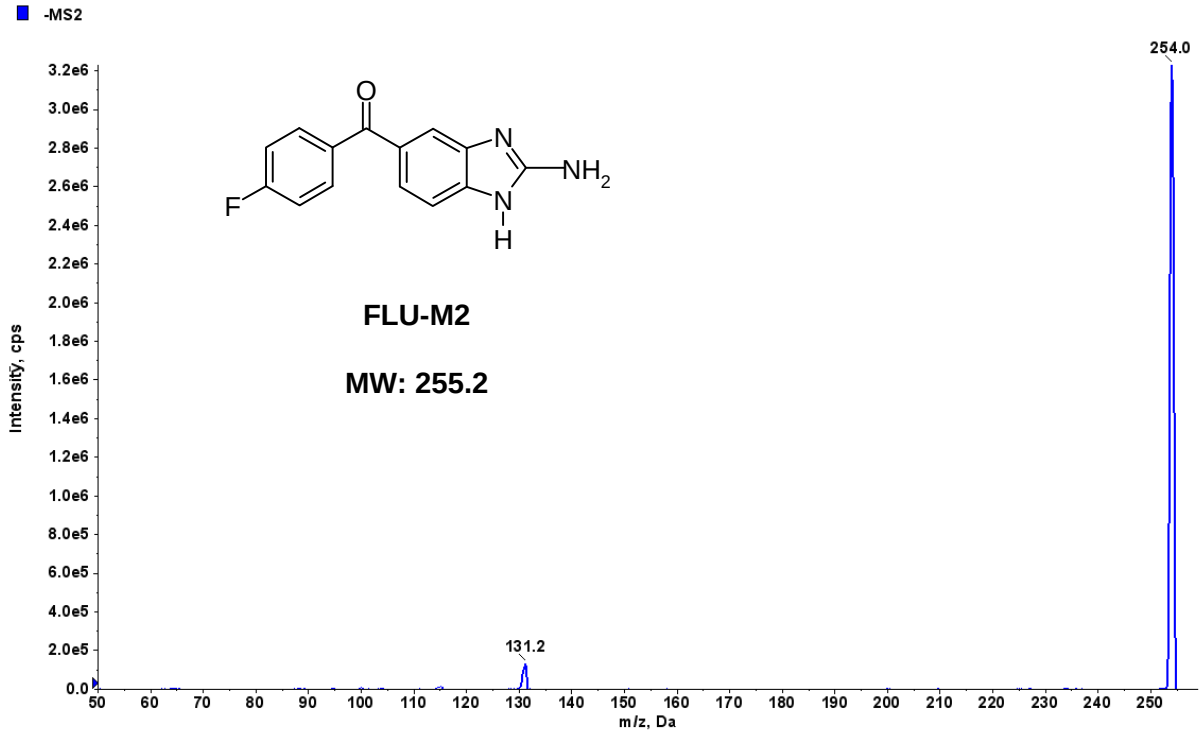
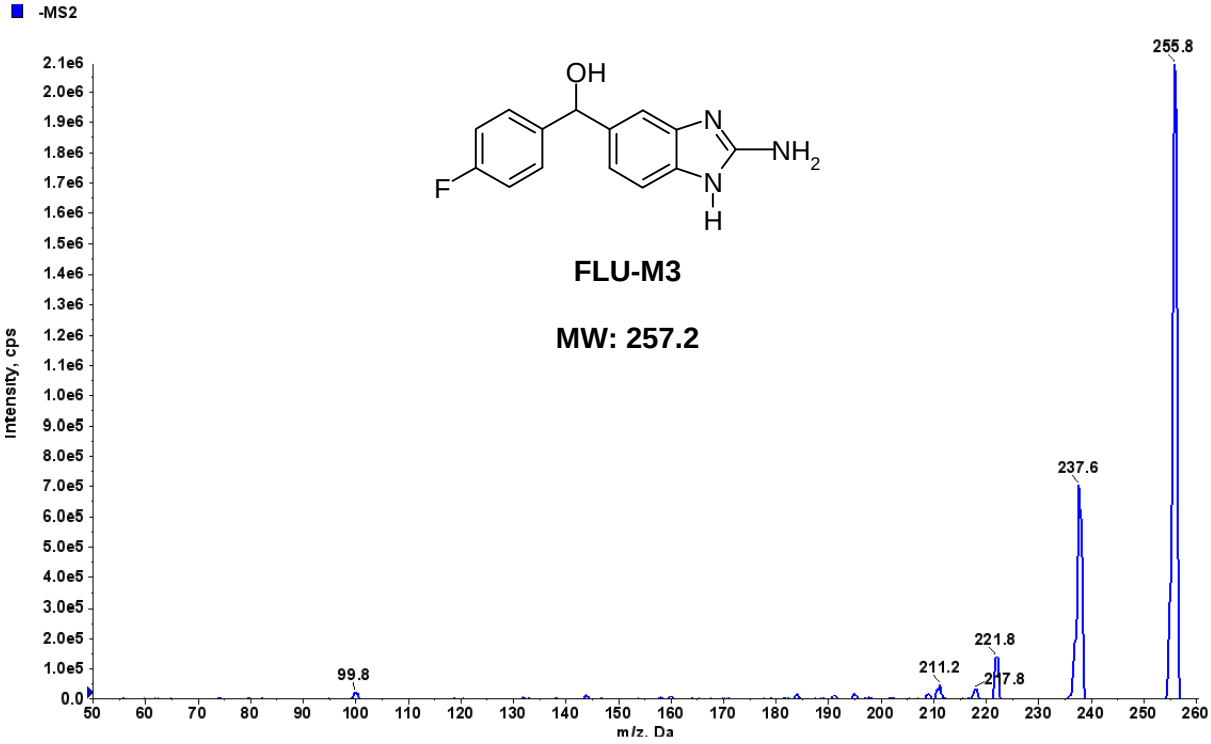
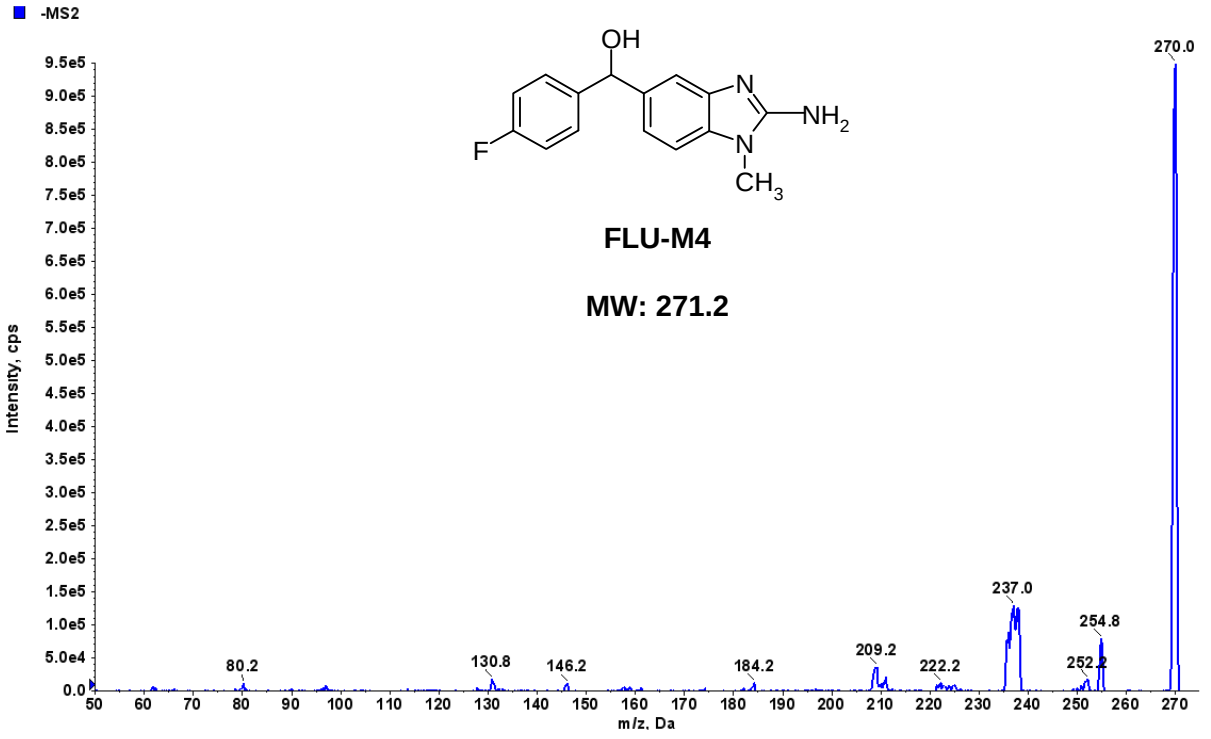


Figure A3: Continued.



FLU-M3

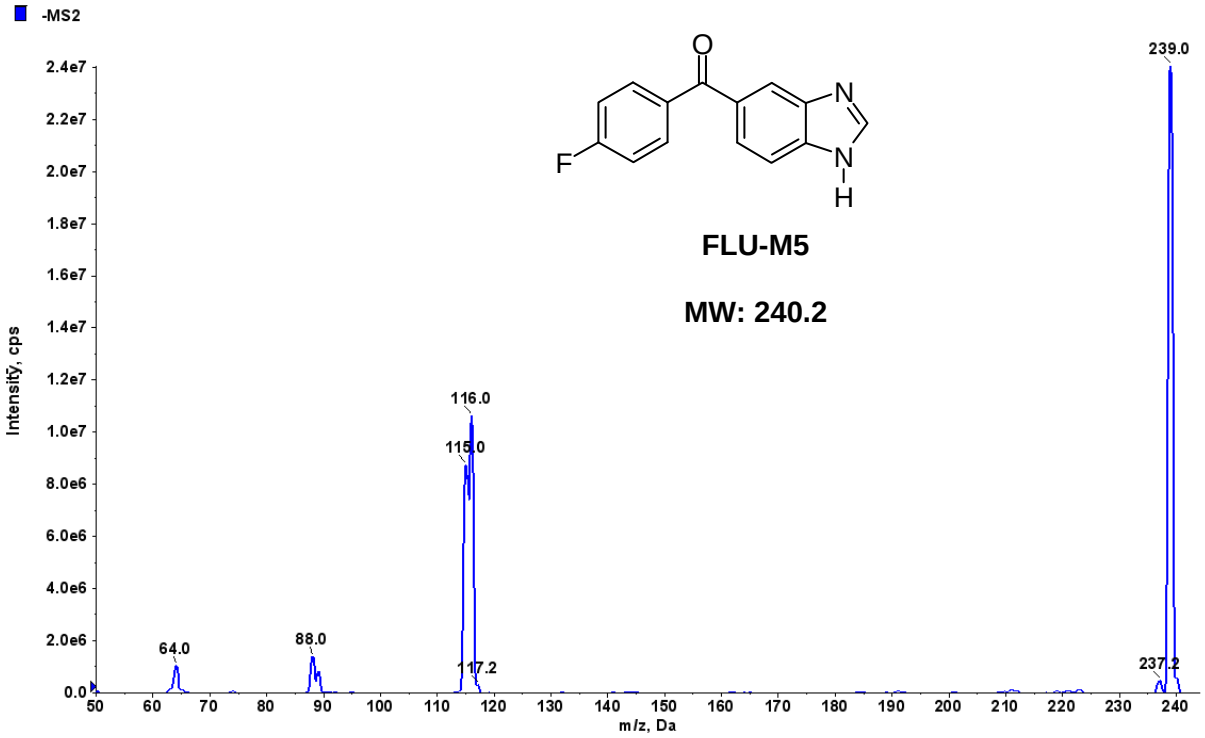
MW: 257.2



FLU-M4

MW: 271.2

Figure A3: Continued.



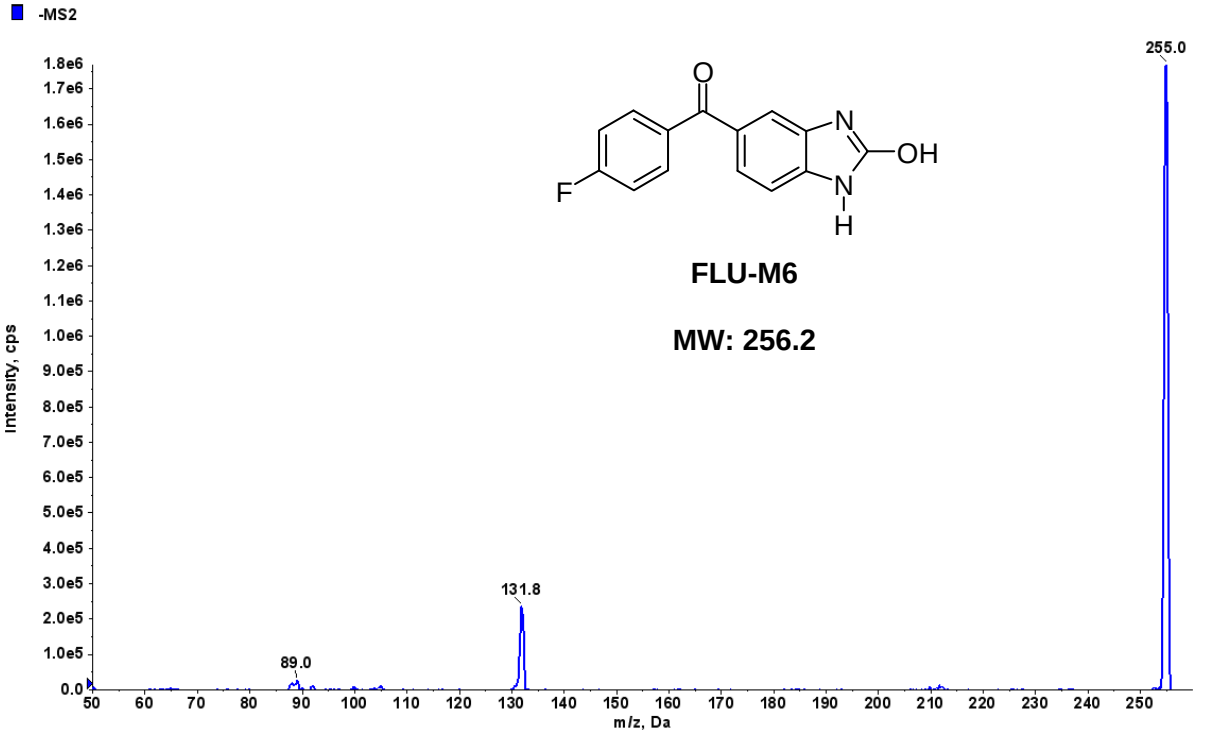


Figure A3: Continued.

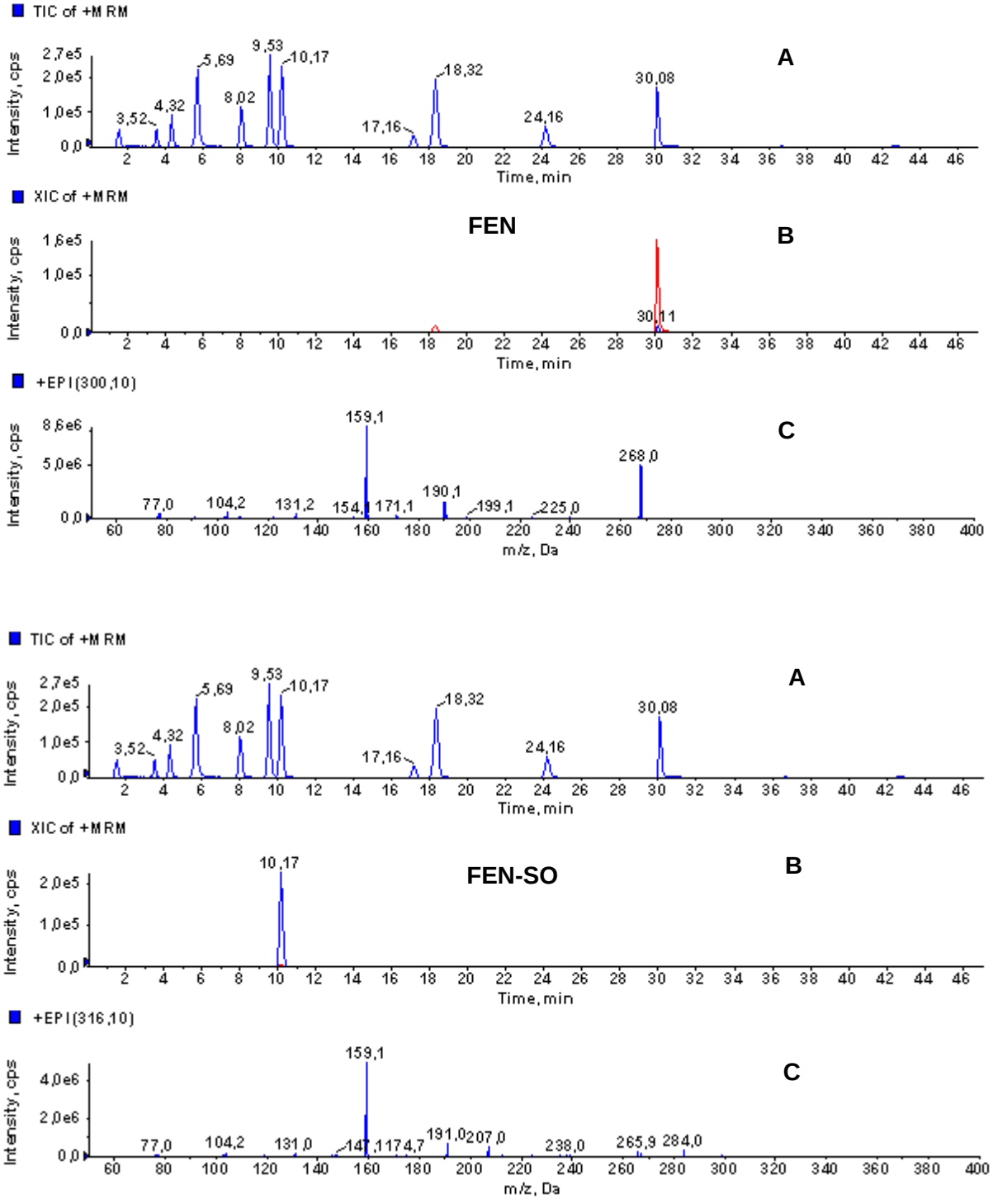


Figure A4: A: total ion current chromatogram of the target compounds (200 pg/µL in methanol), B: extracted ion chromatogram and C: enhanced product ion spectrum obtained using information dependent acquisition method (IDA).

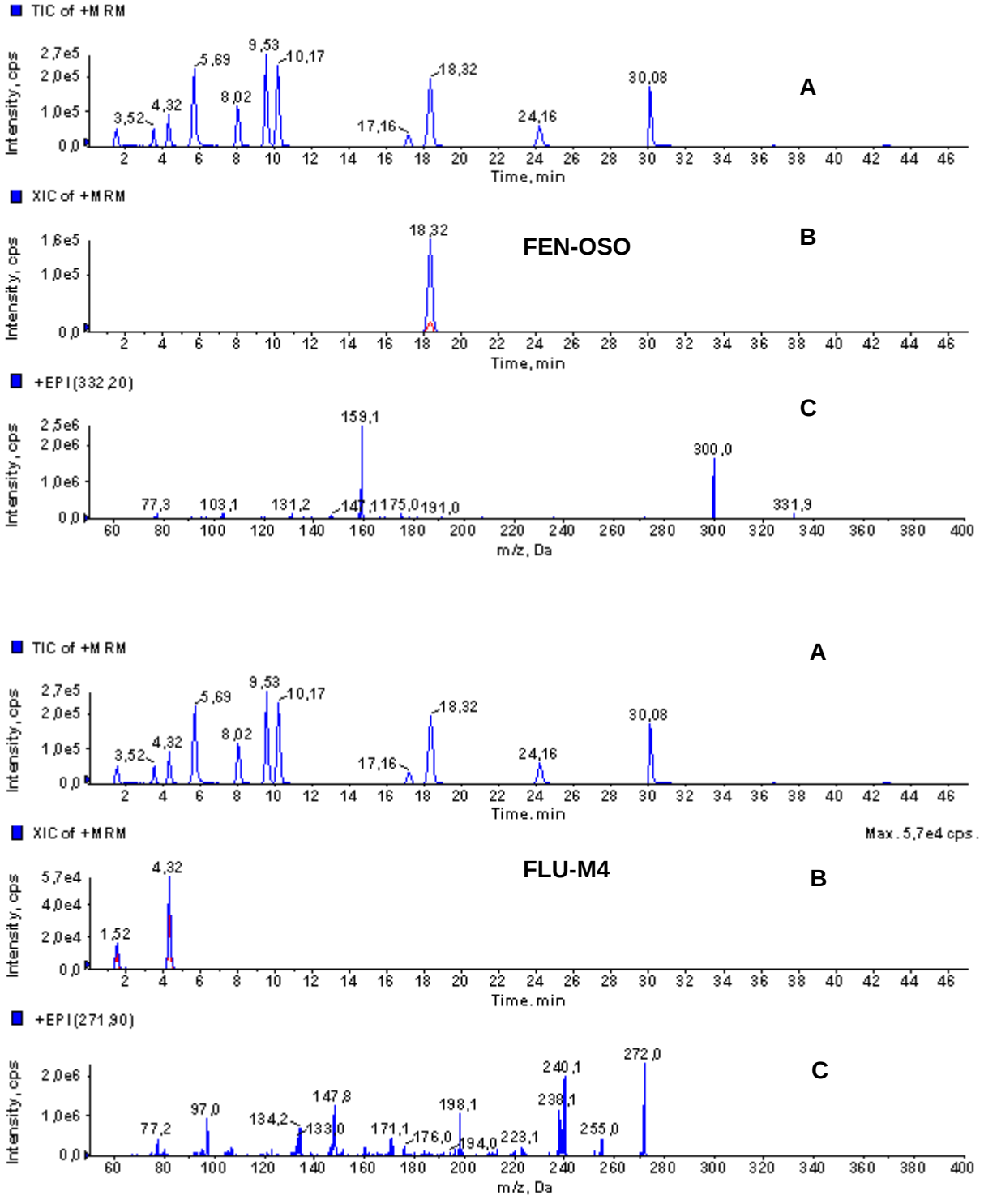


Figure A4: Continued.

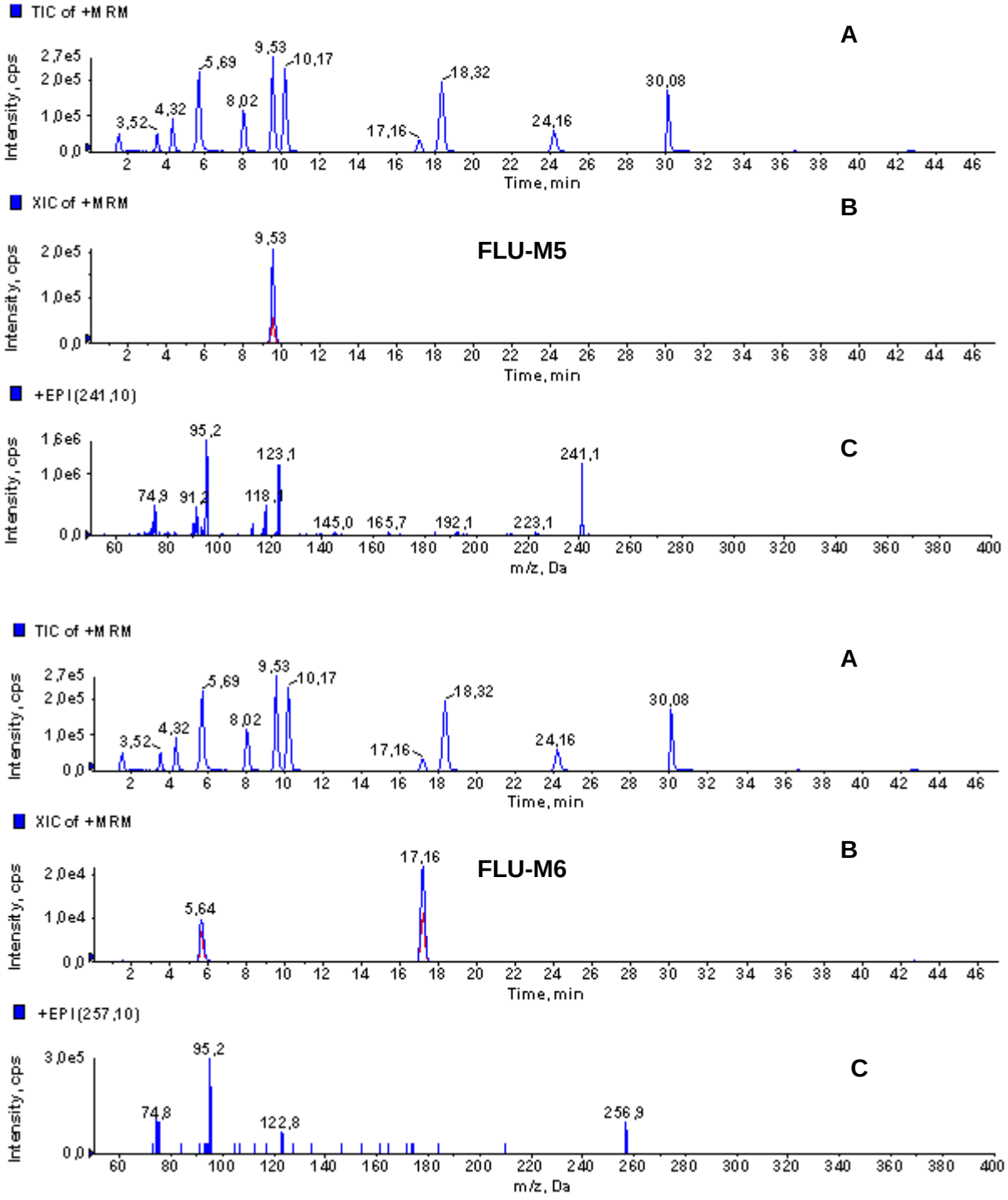


Figure A4: Continued.

Table A1: Recovery rates of fenbendazole, flubendazole with corresponding metabolites spiked into liquid manure samples and directly extracted using ethyl acetate at different pH and cleaned-up using size exclusion chromatography in the preliminary work.

Target compounds	pH 6.8 ± 0.1				pH 4.4 ± 0.1	pH 9.5 ± 0.1		
	[4µg/kg] (n = 7)	[50µg/kg] (n = 3)	[100 µg/kg] (n = 4)	[500 µg/kg] (n = 3)	[100µg/kg] (n =4)	[2µg/kg] (n = 7)	[20µg/kg] (n = 12)	[200µg/kg] (n = 10)
FEN	89 ± 7	89 ± 6	90 ± 4	92 ± 16	88 ± 3	86 ± 19	96 ± 6	80 ±10
FEN-SO	88 ± 9	95 ± 6	98 ± 2	98 ± 6	87 ± 5	94 ± 11	101± 8	89 ± 8
FEN-OSO	97 ± 8	97 ± 5	100 ± 2	100 ± 5	86 ± 3	90 ± 11	100 ± 8	92 ± 8
FLU	87 ± 7	95 ± 6	96 ± 2	101 ± 6	86 ± 3	92 ± 17	98 ± 7	97 ± 7
FLU-M1	87 ± 8	95 ± 6	97 ± 6	83 ± 9	85 ± 4	96 ± 13	94 ± 6	90 ± 7
FLU-M2	75 ± 16	82 ± 8	84 ± 3	95 ± 6	69 ± 5	98 ± 14	95 ± 8	93 ± 12
FLU-M3	74 ± 18	66 ± 6	55 ± 12	63 ± 13	25 ± 10	83 ± 12	92 ± 9	86 ± 7
FLU-M4	79 ± 18	72 ± 11	71 ± 8	78 ± 10	54 ± 4	93 ± 17	94 ± 7	90 ± 7
FLU-M5	83 ± 9	94 ± 6	95 ± 6	101 ± 4	85 ± 4	99 ± 12	98 ± 7	91 ± 6
FLU-M6	88 ± 5	97 ± 9	94 ± 1	99 ± 5	83 ± 4	93 ± 6	98 ± 8	91 ± 6

Table A2: Confirmed results in PM2 according confirmation criteria in EU Commission Decision 2002/657 (EC, 2002).

Target compounds	MRM transitions (amu) (4 IP)	MRM ion ratios in standard solution (n=5)*	Permitted tolerance [%]	MRM ion ratios in real sample (n=2)*	Deviation [%]	Result
FLU	314.20 - 282.20 314.20 - 95.10	0.23	± 25	0.23	0	Confirmed
FLU-M1	316.20 - 284.20 316.20 - 97.20	0.21	± 25	< MQL		< MQL
FLU-M2	256.00 - 95.00 256.00 - 123.10	0.14	± 30	0.15	7	Confirmed
FLU-M3	258.10 -134.10 258.10 - 240.30	0.27	± 25	< MQL		< MQL

*RSD ≤ 2 %

Table A3: Confirmed results in PM3 according confirmation criteria in EU Commission Decision 2002/657 (EC, 2002).

Target compounds	MRM transitions (amu) (4 IP)	MRM ion ratios in standard solution (n=5)*	Permitted tolerance [%]	MRM ion ratios in real sample (n=3)*	Deviation [%]	Result
FLU	314.20 - 282.20 314.20 - 95.10	0.23	± 25	0.23	0	Confirmed
FLU-M1	316.20 - 284.20 316.20 - 97.20	0.20	± 25	0.18	10	Confirmed
FLU-M2	256.00 - 95.00 256.00 - 123.10	0.17	± 30	0.16	6	Confirmed
FLU-M3	258.10 - 134.10 258.10 - 240.30	0.31	± 25	0.29	7	Confirmed

*RSD < 5 %

Table A4: Confirmed results in PM4 according confirmation criteria in EU Commission Decision 2002/657 (EC, 2002).

Target compounds	MRM transitions (amu) (4 IP)	MRM ion ratios in standard solution (n=5)*	Permitted tolerance [%]	MRM ion ratios in real sample (n=3)*	Deviation [%]	Result
FLU	314.20 - 282.20 314.20 - 95.10	0.23	± 25	0.27	15	Confirmed
FLU-M1	316.20 - 284.20 316.20 - 97.20	0.18	± 25	0.19	6	Confirmed
FLU-M2	256.00 - 95.00 256.00 - 123.10	0.14	± 30	0.15	7	Confirmed
FLU-M3	258.10 - 134.10 258.10 - 240.30	0.27	± 25	0.29	7	Confirmed

*RSD ≤ 5 %

Table A5: Confirmed results in PM5 according confirmation criteria in EU Commission Decision 2002/657 (EC, 2002).

Target compounds	MRM transitions (amu) (4 IP)	MRM ion ratios in standard solution (n=5)*	Permitted tolerance [%]	MRM ion ratios in real sample (n=2)*	Deviation [%]	Result
FLU	314.20 - 282.20 314.20 - 95.10	0.23	± 25	0.22	4	Confirmed
FLU-M1	316.20 - 284.20 316.20 - 97.20	0.20	± 25	0.17	15	Confirmed
FLU-M2	256.00 - 95.00 256.00 -123.10	0.17	± 30	0.15	12	Confirmed
FLU-M3	258.10 - 134.10 258.10 - 240.30	0.31	± 25	0.29	0	Confirmed

*RSD ≤ 3 %

Table A6: Confirmed results in PM6 according confirmation criteria in EU Commission Decision 2002/657 (EC, 2002).

Target compounds	MRM transitions (amu) (4 IP)	MRM ion ratios in standard solution (n=5)*	Permitted tolerance [%]	MRM ion ratios in real sample (n=2)*	Deviation [%]	Result
FLU	314.20 -282.20 314.20 - 95.10	0.21	± 25	0.22	5	Confirmed
FLU-M1	316.20 -284.20 316.20 - 97.20	0.18	± 25	0.17	6	Confirmed
FLU-M2	256.00 - 95.00 256.00 -123.10	0.13	± 30	0.1 3	0	Confirmed
FLU-M3	258.10 -134.10 258.10 - 240.30	0.28	± 25	0.29	4	Confirmed

*RSD ≤ 9 %

Table A7: Confirmed results in PM7 according confirmation criteria in EU Commission Decision 2002/657 (EC, 2002).

Target compounds	MRM transitions (amu) (4 IP)	MRM ion ratios in standard solution (n=5)*	Permitted tolerance [%]	MRM ion ratios in real sample (n=3)*	Deviation [%]	Result
FLU	314.20 - 282.20 314.20 - 95.10	0.18	± 25	0.18	0	Confirmed
FLU-M1	316.20 - 284.20 316.20 - 97.20	0.20	± 25	0.19	5	Confirmed
FLU-M2	256.00 - 95.00 256.00 -123.10	0.12	± 30	0.12	0	Confirmed
FLU-M3	258.10 -134.10 258.10 - 240.30	0.26	± 25	< MQL		< MQL

*RSD ≤ 10 %